

Many catastrophes too many

The concatenation of human catastrophes in the past few months has found the world unable or unwilling to deal with them: does realism suggest they are beyond aid?

THE recent list of human disasters seems endless and may literally be so. Several million people have been displaced by the Gulf War, many dying *en route*; the casualties of the war have not yet been counted. Some thousands have less conspicuously died of cholera in Latin America, more than 200,000 Bangladeshis were swept to their deaths by the typhoon two weeks ago and now there is the tragic prospect that the plight of the 27 million people at risk from starvation in sub-Saharan Africa will be worsened by the flight of more than a million people across the Somali border into Ethiopia, of all places. All these claims on the rich world's compassion have overwhelmed its capacity or its willingness to respond. What has gone wrong?

Government cutbacks

Ten years have passed since Dr Willy Brandt's commission (best known for calling the developing countries of the world "the South") urged that rich countries should devote one per cent of their Gross Domestic Product (GDP) to development assistance, but the trend has been in the opposite direction. With the honourable exceptions of Japan (now spending close on \$10,000 million a year), the Scandinavian states, the Netherlands and Canada, most governments have been cutting back in the hope of solving politically more pressing domestic problems. The disparity between what governments spend at home and abroad is amply illustrated by the pre-election row in Britain between the government and its chief political opponents; the government would double the extra £20,000 million the opposition promises to spend on domestic social services, but this extra is at least twenty times the official British aid budget. No wonder that the gap between the rich and poor countries is widening steadily. It will get wider: the Brandt commission's plea for one per cent of every rich country's GDP is much less than the need.

What is the need? One of the few benefits of the present overloading of the rich world's ramshackle way of raising money for the poor world — a prominent British author/politician, Mr Jeffrey Archer, hopes to raise £20 million for Kurdish refugees by organizing a popular music concert in a football stadium — is that it may force an acknowledgement that the time has gone for palliation. Sacks of flour sent to the Sudan, tents, blankets and penicillin sent to Bangladesh and electrolyte-replacement kits sent to Peru may save a few lives now, but will not prevent the recurrence of catastrophes that are, in any case, avoidable. In the rich world, construction standards and public health regulations abate the damage

done by hurricanes and infection, but rich countries have forgotten that it is a luxury, sustainable only by prosperity, to enjoy and then enforce such defences against natural calamity. So the long-term remedy is to make the poor world prosperous as well.

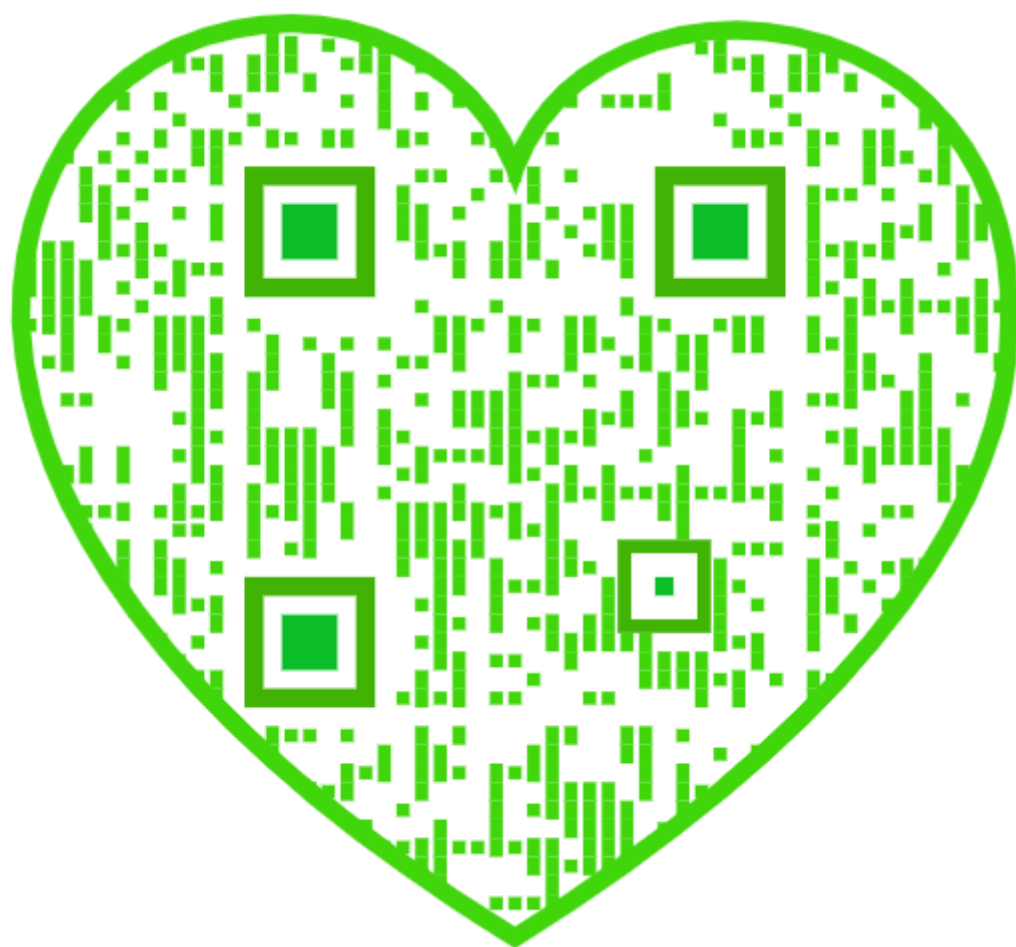
That is a tall order, but not a new one. For decades, development rather than assistance has been the slogan among farsighted governments and organizations such as the World Bank. India has done better than most people elsewhere allow with the funds provided under this rubric. South Korea and, to some extent, Brazil have managed to make good use of private capital for development. Mexico, never quite on its beam-ends, is now on the road to self-sustaining development. Until two years ago, China seemed full of promise. But for most of the poor countries of the world, these recipes are unattainable. And the reasons are not far to seek.

For one thing, the scale of development assistance has never matched the need. If two-thirds of the world's population lives below an abysmal poverty line, simple arithmetic shows that rather more than two-thirds of the world's investment must be shunted in their direction if the balance is ever to be redressed. But most of the countries of Latin America and Africa are saddled with external debts they can repay only by mortgaging their meagre export earnings, and so can do very little from their own resources. Indebtedness has also frightened away private capital, which in any case has better (or more profitable) investments to make. That this should have come at a time when the rich world, already strapped for capital, is hard-pressed to scrape together the funds to revivify Eastern Europe is a cruel misfortune for the poor world.

Trade restrictions

In the long run, the only durable solution is trade. If the world's poor countries are ever to be freed from demeaning dependence on singalongs in the football stadiums of the industrialized West, they will have to be enabled to keep themselves and find the capital they need by selling what they can elsewhere. That is why it is such a cruel disappointment that even the still-unrequited attempt at a further liberalization of world trade under the General Agreement on Tariffs and Trade (GATT) continues to include repressive restrictions on the poor world's textile trade. Nothing has yet been done to negotiate means by which poor countries might trade their way out of trouble by selling what they can elsewhere.

Another misfortune is that much of the poor world is saddled with governments whose incompetence is often



matched by their willingness to put narrow and personal self-interest and ideological causes before their peoples' needs. Recurrent famine in Ethiopia in the past decade owes much to the government's hostility to the grain trade's middlemen, who may have traditionally exploited poor farmers but who at least kept the grain moving. In the Sudan, where many of those now at risk from famine try to live, orthodox Muslim Khartoum believes that the dissenting south is both heretical and treasonable, and that the war against dissent takes precedence over that against hunger. Meanwhile, the ferocity of Liberia's civil war, which chilled the spines of our global village only the other day (last year), has almost been forgotten.

Public incompetence

Good government is indeed a necessary condition for survival; how else can public health regulations be promulgated, let alone enforced? (Why bother with safe construction standards until there are enough dwellings to go round?) And what else can ensure that funds intended for development do not end up in Swiss bank accounts? In the tumult following the Gulf War, there has been some heady talk of how the international community, having set up a safe haven for Kurdish refugees in Iraq, might go further and step in wherever public incompetence seems to threaten private tragedy. But that is wishful thinking. The United Nations charter, based on the sovereignty of sovereign powers, will not be substantially amended for a while. The only remedy, at this stage, is that incompetent and corrupt governments should be told what their faults are in the plainest language. The voluntary aid agencies, dependent as they often are on the tolerance of the governments whose people they try to help, are not well placed to do the job. The responsibility falls on other governments, too many of which are ready to bite off their tongues for immediate political advantage.

A further difficulty is the irresolution that has afflicted the development assistance community in the past few years. The notion of 'appropriate technology' has a lot to answer for: it is as if the poor world is being told that it must begin, as did industrial Europe, by building watermills so that, afterwards, it may graduate to the manufacture of, say, internal combustion engines.

To the extent that many of the particular problems of poor countries, especially in Africa, arise because their populations have outstripped the traditional carrying capacity of the land they live on, it seems pointless now to be seeking extensions of traditional ways of making life sustainable. Why not help poor countries to leapfrog over their equivalent of Europe's dark ages? The notion that all development must now be green is similarly a drag on what can be done. As the industrialized world bends to cleaning up past messes, should not the poor world be allowed a few of its own if the stake is its survival?

The irresolution is debilitating because it prevents those who administer development funds from forming a vision of where success could lead, which then reinforces the general impression that the cause of development in the poor world is like that of using a teaspoon to fill a bottomless pit. In reality, there is better cause for hope. For one thing, not all poor

countries are alike; some are better governed than others, some are better endowed with raw materials and other natural resources.

More to the point, there is little to distinguish between the civility of the poor and the rich countries except the dozen or so generations of rapid industrial development that the division of labour has given the rich world. Who says that what was possible in Europe in the eighteenth and nineteenth centuries cannot elsewhere be repeated?

Chiefly the cataclysmic millennialist prophets who now abound, with several interlocking arguments mostly linked with the pace of growth of the poor world's population. The malthusian fear that population growth means famine, current as recently as the World Food Conference in 1970, has been banished by the embarrassment of agricultural surpluses around the world, but population growth is as much a symptom as a cause of poverty. When children die like flies from intestinal infections, who can blame subsistence farmers for overbreeding? And which neocolonialist says that Europe's demographic transition to low birth and death rates in the nineteenth century is unattainable elsewhere? The reality is that many now-poor countries will eventually be as free from the cruelties of their natural environment as are the industrially rich countries now. And others, on present form, will sink without trace.

So what to do about tomorrow's calamities? Enlightened development, however adventurous, will not prevent needless death here and now.

So there is a balance that must be struck between the present and the future. The calculation that the future is the more important is too callous for many people and, sadly, calamities wring money from rich pockets more readily than long-standing needs, however glaring. But the compassion widely stirred in the past few months should be tempered by the knowledge that it is palliation, not a cure. The best outcome would be that the flood of fellow-feeling that promises to fill the football stadiums, and the general sense that there are now more calamities than can be handled, will combine to impel rich governments towards more enlightened policies.

Development assistance

What should they be? The United Nations charter may never be amended, nor should it be on these grounds. (There is a better case for the attenuation of sovereignty in the interests of controlling the spread of some military technology.) But that does not leave rich governments powerless. Would it not make sense that they should insist, in their own development assistance and that provided by the international organizations to which they belong, that recipient governments themselves follow policies likely to be conducive to development? On present form, the pattern of spending would be greatly changed. Latin America would generally profit, with tiny Costa Rica near the top of the pile. Africa, the graveyard of past hopes, would by comparison do less well. And if the outcome should be an international simulation of the *triage* practised by physicians on the battlefield, who is to say that it is unjust? □

US getting little help in funding the SSC

- Few commitments expected in near future
- Questions remain about final price tag

Washington

HOPES are rapidly evaporating that other countries will offer substantial financial help to the United States in building the Superconducting Super Collider (SSC), judging from testimony given at a hearing of a House of Representatives subcommittee last week. This would leave Congress with the uncomfortable choice of either providing another \$1,000–\$2,000 million to pay for the giant particle accelerator or cancelling the project.

At the hearing, congressmen sharply questioned officials from the Department of Energy (DOE) on the status of the SSC, dwelling on the DOE's cost estimate of \$8,249 million and on whether the SSC can stick to a rigid construction schedule that makes little allowance for unforeseen setbacks. For instance, subcommittee chairman Howard Wolpe (Democrat, Michigan) pointed out that the estimated cost does not include certain funds necessary for finishing the SSC, such as money for spare parts and for operating the SSC laboratory while it is under construction, and these funds bring the SSC's total cost to \$9,100 million. Furthermore, Joseph Cipriano, DOE's project manager for the SSC, testified that every year of delay in construction will add \$400 million to the SSC's cost.

The DOE officials argued that their cost estimates and construction timetable are reasonable and are being held to. They had little defence, however, against concerns that foreign contributions are nowhere in sight, because the House subcommittee was able to produce an internal DOE report that conceded as much.

DOE had spoken to possible foreign collaborators, the report said, and concluded that "we [DOE] believe that we are unlikely to meet the Administration's goal for non-federal participation (one-third of the total project cost) in the foreseeable future." Garry Gibbs, of DOE's Office of the SSC, told the subcommittee that the only firm commitment so far is a \$50 million in-kind contribution from India. He acknowledged that the department does not expect much money from Europe but said that DOE is negotiating with non-European countries, such as the Soviet Union and Brazil, and is hoping for a major contribution from Japan.

The Japanese, however, are in no rush to make a decision, the internal DOE report says, and it will probably take a direct appeal from President George Bush to Japanese Prime Minister Toshiki Kaifu to get Japan to make a pledge. Since June 1990, when Bush officially invited Kaifu to join the project, the two have had two summit meetings, and the

subject of the SSC has not been raised. Japanese officials "have interpreted this as a sign of the Administration's priorities," the report states, and thus feel in no hurry to commit themselves.

Originally, DOE predicted that other countries would become involved in the SSC project early on and would make in-kind contributions — helping with planning, magnet construction and the like. Now the department says that other countries are unlikely to commit themselves to the SSC until the United States commits itself, and so most foreign help will probably come towards the end of the project and will be in cash. This creates a new problem.

DOE's official cost schedule, which outlines funding and expenses for the project, relies on early in-kind contributions from foreign sources — \$670 million by 1995. If other countries do not make contributions by then, either Congress will itself have to provide the extra money or the construction schedule will have to be extended — at a cost of \$400 million for each year of delay.

Congressman Wolpe noted that DOE seems to have used rather 'creative' accounting methods in its cost estimates. One example was its projection of how much foreign money is needed to meet the department's goal of one-third outside participation. DOE took the total estimated project cost, divided by three and then subtracted the \$875 million pledged by the state of Texas as well as \$219 million in foreign contingency costs — money needed for unexpected cost overruns for in-kind contributions, such as magnet-building by Japan, for instance.

But as DOE acknowledges that it now expects few in-kind contributions, foreign contingency costs should not be present, Wolpe said. Furthermore, he noted, DOE had already subtracted the (probably inflated) foreign contingency costs out of its total project cost in order to make that figure as low as possible, so subtracting them from the foreign contribution meant that they were subtracted twice. The DOE contingent did not explain the rationale behind the accounting methods.

The final decision either to finish the SSC or to abandon it will probably not have to be made this year, but it seems increasingly likely that Congress will have to make up its mind without knowing just how much of the cost other countries will pick up. Wolpe summed up this dilemma by saying that the SSC project appears to have only two stages: too soon to tell, and too late to stop.

Robert Pool

INDIRECT COSTS

Flowers, opera and art

Washington

FOR US university scientists, who have long complained that administrators are living well off research accounts, last week's congressional hearing on university overcharging may have been the best revenge. Government investigators revealed that university presidents and their spouses had flown to Caribbean resorts, thrown parties, bought opera tickets and made donations to art museums, all in the name of research, at least as far as their accounting records went. The investigators are challenging more than \$14 million of suspect costs at 13 universities — part of a government investigation that will expand this week to 270 other universities.

Among other items improperly charged to research accounts, investigators found wine glasses, jewellery, cigars, a sculpture that had already been donated by a patron, flowers, golf clubs, private club dues, party balloons and liquor.

The 13 universities identified last week were Dartmouth, Yale, Rutgers, Johns Hopkins, the University of Pennsylvania, the University of Pittsburgh, Duke, Emory, the University of Michigan, the University of Chicago, the University of Texas Southwestern Medical Center at Dallas, Washington University and the University of Southern California. They join seven others already under investigation. Among those seven, Stanford, Harvard University Medical School, the Massachusetts Institute of Technology and California Institute of Technology have so far agreed to return a combined total of \$2.7 million in improper charges.

Last week, two congressional committees made the first legislative attempts at reforms. Rick Boucher (Democrat, Virginia) introduced legislation that would limit total indirect costs to 45 per cent. And Henry Waxman (Democrat, California), chairman of the House Energy and Commerce health subcommittee, included a 26 per cent cap on the administrative portion of indirect costs in his proposed reauthorization legislation for the National Institutes of Health.

Both proposed legislative limits are at about the average current level of indirect costs. Although such a measure might appear to produce little in the way of savings, NIH have calculated that declaring even the current average to be an absolute ceiling could save \$100 million by forcing universities over the limit to reduce their charges. Although neither the Waxman nor the Boucher bill is considered likely to pass (Waxman's bill, for example, is endangered by its attempt to overturn the Administration's fetal tissue research ban), the powerful House Appropriations Committee plans to include an indirect cost cap in the NIH budget later this year.

Christopher Anderson

Spat over Gulf War windfall

Washington

IN winning the war in the Persian Gulf, US military officials were forced to surrender, at least temporarily, in another dispute — a long-running struggle with researchers over access to satellite data. But now that the war is over, the Department of Defense is threatening once again to take back its data, and the researchers are up in arms.

Early last year, scientists protested when the Department of Defense announced a decision to begin encrypting the signals from its array of Global Positioning System (GPS) satellites. With their ability to give precise Earth coordinates in real time, these satellites have become irreplaceable tools for an increasing number of researchers. Although encrypted signals are still usable for some applications, encryption reduces the position accuracy by almost a factor of ten for anyone who does not have the special receivers that decode the signals. So scientists — including some Japanese seismologists who found themselves suddenly unable to measure crustal movement to predict earthquakes — criticized the decision and sought special access for legitimate research uses (see *Nature* 345, 195; 17 May 1990).

But the squabble ended abruptly late last year when the researchers discovered that the encryption had been mysteriously turned off. And after a little investigation, they dis-

covered the reason: GPS had proved so useful as a navigational aide to the troops that the demand for military receivers (with decryption) quickly outstripped the supply.

The only quick solution was for Allied forces to buy the same commercial receivers that scientists have been using for geological and Earth-observation research — and for the Department of Defense to return GPS to its original open-access status so that the commercial receivers could be used.

Researchers fear, however, that the lifting of the signal encryption may be only temporary. While the military now owns thousands of commercial GPS receivers that would be nearly useless if the encryption — known as selective availability — were turned back on, the war is, after all, over and those receivers are not now needed. Department of Defense officials argue that if they leave the encryption off in peacetime, commercial systems — and perhaps even GPS-guided enemy missiles — will proliferate.

"If full GPS accuracy is available to the public... there will be irresistible pressure to maintain signal accuracy in time of crisis," US Air Force General Donald Kutyna told the Senate Armed Forces Committee last month. "I believe that would place our forces at risk from smart weapons using GPS guidance."

A military review panel has been assem-

bled to make recommendations on when and how much GPS distortion should be used in peacetime. The GPS Association, a California-based coalition of GPS receiver manufacturers, is lobbying Congress and the Department of Defense to keep the encryption turned off permanently. Both the American Congress on Surveying and Mapping and the American Geophysical Union (AGU) are also arguing in favour of full civilian access.

At stake for science is mostly the time and money of researchers who now use GPS and of the increasing number of those who are discovering the utility of having a \$3,500 receiver that can give an absolute global position within five metres. With encryption turned on, real-time measurements degrade to an accuracy of between 30 and 100 metres. Although researchers can use multiple receivers and additional processing of the signals to regain much of the original accuracy, the extra equipment and time can add thousands of dollars to a project.

In the case of ocean floor mapping, where one shipboard receiver gives all the accuracy needed as long as the encryption is off, logistics quickly become a nightmare when it is turned on. To maintain a five-metre accuracy in the face of signal encryption, researchers have to place a second receiver on the closest shore, set up a radio and computer link between the two, and then constantly move the land-based receiver to stay in range.

Christopher Anderson

Magellan gets set to fill in Venus's gaps



MAGELLAN, NASA's space probe orbiting Venus starts the second cycle of its mission today (16 May). Since 16 August last year, Magellan's 3½ hour polar orbit has taken it over the whole of the surface of Venus. Eighty two per cent of the surface has now been mapped: some data were lost while Venus was hidden on the far side of the Sun; and mapping of the planet's south pole was impossible owing to the probe's orientation. The picture above shows a three-dimensional perspective view of part of one of Venus's highlands, reconstructed

from the orbiter's radar data. Lakshmi Planum, to the right of the image, has an average elevation of 2.5 to 4 km; the mountain to the rear, Danu Montes, rises a further 1.5 km. The gaps in Magellan's coverage, seen above as simple black strips, should be filled in during the probe's second 243-day cycle. A change in the probe's orientation (looking eastwards rather than westwards) will enable it to map the southern pole. One aim is to find evidence of change in regions identified as geologically active during the first pass.

Roland Pease

Door opens for national labs

Tokyo

A BUREAUCRATIC wall that has prevented effective collaboration between researchers in Japan's universities and national research laboratories is about to crumble.

In Tsukuba science city north of Tokyo, a newly proposed scheme will allow researchers from 30 national research institutes affiliated to seven different government agencies and ministries to take up positions as visiting professors or assistant professors at Tsukuba University in order to supervise graduate students. To Western eyes, this may seem unremarkable, but in Japan it is almost revolutionary and indicates a new willingness on the part of Japan's various government agencies and ministries to cooperate in research.

Until very recently, the Ministry of Education, Science and Culture (MESC), which runs Japan's national universities, has steadfastly guarded its territory and refused to allow non-MESC researchers to participate in university research. In particular, the supervision of graduate students has been strictly limited to university faculty members.

This damaged Japanese research in at least two ways. First, the national laboratories have not been able to bring in new, young researchers, as clampdowns on hiring have combined with the unavailability of graduate student workers to magnify the effect.

Second, graduate students in universities have not had access to the newer, better facilities of the national laboratories and have had to make do with the often outmoded equipment of the universities.

Now, Tsukuba may open the way for graduate students to work with scientists from the national laboratories. Although Tsukuba University is the only exception so far to MESC's rules, all the major national research institutes have locations in Tsukuba and thus will have access to graduate students there. And some observers see the recent move as a sign that MESC is becoming more flexible.

The first crack in MESC's bureaucratic wall actually appeared in 1988, when the ministry established a new graduate university where researchers from MESC national research institutes could supervise graduate students and award degrees (see *Nature* 333, 290; 1988). There are only a handful of MESC institutes involved in the graduate university, however, and the ministry still refused researchers from other national laboratories the right to train graduate students. Another breach was made last year when MESC allowed Saitama University to appoint visiting professors and assistant professors from the Institute of Physical and Chemical Research (RIKEN) in Wako city in the outskirts of Tokyo — RIKEN is a 'special corporation' (*tokushuhogin*) affiliated to the Science and Technology Agency that enjoys unusual freedom from bureaucratic control. Once again, however, this involved only a few researchers, and as RIKEN is not a normal national laboratory, this move did not automatically imply an opening for other institutes.

Nonetheless, Yoshimasa Yoshizawa, vice president of Tsukuba University, says that the RIKEN-Saitama agreement was the 'driving force' for the new Tsukuba scheme. And he says he was pleasantly surprised by the ease with which MESC accepted Tsukuba's plan. "Five years ago it would probably have been a lot more difficult," he says.

Under the scheme, about 50 researchers from Tsukuba national laboratories will be appointed to the university from next fiscal year and will each supervise one or two graduate students in their respective laboratories. A handful of researchers will also be drawn from private company research laboratories in Tsukuba, including institutes belonging to Takeda and Eisai pharmaceutical companies. But although there are now 140 private-sector laboratories in Tsukuba, Yoshizawa does not expect many companies to join the scheme. Most private companies, he says, do not wish to disclose their proprietary research results.

Yoshizawa says that when government officials planned Tsukuba science city in the 1960s, they expected some interaction between the closely grouped government research laboratories and Tsukuba University. But in fact, although many researchers have informal contacts through research clubs and societies, very little formal cooperative research has been possible because of the bureaucratic barriers between the various ministries and agencies that run research organizations in Tsukuba.

The scheme has yet to get formal approval from MESC and the Ministry of Finance.

David Swinbanks

ACID RAIN

An economic case for environmental cooperation

London

WITHOUT an unprecedented degree of economic co-operation, the countries of Europe could collectively waste more than \$20,000 million a year in relieving the damaging effects of acid rain across the continent, according to an economic analysis done by Bo Döös, deputy director of the International Institute for Applied Systems Analysis (IIASA).

Merely to keep the damage of acid rain across the continent from getting worse, Europe's sulphur dioxide emissions must be cut by 60–80 per cent, Döös says. This could be achieved in a number of ways. The simplest — each country cutting its emissions by some 70 per cent — would cost some \$31,000 million a year. But the same result could be achieved for only about \$9,000 million a year, Döös says, by focusing the cuts in emissions more carefully.

In this case, the cuts would be concentrated in those countries that are contributing most to the problem. But these countries, which include the former communist regimes of central and eastern Europe, are also the least able to pay for pollution cleanup and will require substantial economic assistance.

Because the richer countries of Europe will be reluctant to provide this aid, Döös accepts that his lower cost estimate is probably unrealistic. But he does believe that a series of bilateral agreements between European governments could realistically reduce the costs of stabilizing the damage caused by acid rain by 20–30 per cent.

Döös adds that cutting sulphur dioxide emissions will in the long run have net economic benefits — the benefit to forestry alone would be some \$28,800 million a year, across the whole of Europe. But governments may still be unwilling to provide massive aid to cut emissions elsewhere without more tangible proof that they themselves will gain economically by doing so.

Peter Aldhous

National lab researchers dissatisfied

GOVERNMENT researchers in Tsukuba science city may have broken through one bureaucratic barrier (see above), but most young researchers in Japan's national laboratories are still dissatisfied with their lot, according to a survey released last week by the Science and Technology Agency.

The survey was carried out by sending a questionnaire through several electronic mail computer networks used by scientists in Tsukuba and other regions of Japan. Eighty researchers, who are in their thirties and forties and come mainly (75 per cent) from national research laboratories, replied. More than half (43) are from Tsukuba.

When asked if satisfied with their job, 70 per cent said "no". Among the reasons given were: "I cannot do the research I want to do" (18 per cent); "I cannot get satisfactory results easily and have a lot of worries about my research" (16 per cent); and "salary and working conditions are poor" (22 per cent).

More than half of the respondents (56 per cent) said they would gladly move elsewhere if recruited. Among the suggestions of what should be done to improve conditions in the laboratory the most popular reply (30 per cent) was a call for introduction of flexible working hours (flexitime). **D.S.**

Hazards of risk assessment

Washington

THE art of identifying and regulating human health hazards — a ramshackle mix of science, black magic and guesswork, as it is currently practised by the US government — is now moving into the even murkier waters of economics. After years of operating in the grey world of animal models and statistics, officials are beginning to realize that many of their regulations bear little relation to real-world risks. As they attempt to reform the system, they are finding strong arguments for weighing the costs of regulations with their benefits — something that only a decade ago was tantamount to heresy.

Both in regulation and evaluation, "the time is absolutely ripe for re-evaluation", says former FDA commissioner Frank Young, now deputy assistant secretary for health, science and the environment at the Department of Health and Human Services. "We've failed the public in not doing the scientific work of risk assessment." First warning of major changes came last year with a virulent attack on the system from economist Richard Belzer. That an economist would argue for more cost-benefit considerations in risk assessment may not be surprising. But Belzer works for the White House Office of Management and Budget, and his 13-page tirade against current regulatory practices came not in an academic journal, but in the *Regulatory Program of the United States*, the official statement of government policy philosophy.

Belzer minced no words in describing what he saw as a regulatory system crippled by its reliance on flawed assumptions, animal models, and its shortsighted approach to overall risks.

"The continued reliance on conservative (worst case) assumptions distorts risk assessment, yielding estimates that may overstate likely risks by several orders of magnitude", Belzer warned. Such conservatism forces the Federal Government to direct scarce resources "to reduce what are often trivial carcinogenic risks while failing to address more substantial threats to life and health".

As an example of such inequities, Belzer pointed to the banning of EDB, a grain and soil fumigant that combats peanut mould, which can harbour the potent carcinogen aflatoxin B. In part because of the decision to forgo the chemical, the estimated cancer risk from one peanut butter sandwich is now about 75 times that of a full day's dietary exposure to EDB.

Prompted by Belzer's attack on the status quo, the White House Office of Science and Technology Policy set up a panel of representatives from all the regulatory agencies to reconsider the subject. Formed as a subcommittee of the Federal Coordinating Council for Science Engineering and Technology (FCCSET), the panel is charged with ensuring that policies on risk assessment

reflect scientific consensus and are uniformly applied across the government.

That has turned out to be an instructive exercise. One of the first issues to come under FCCSET scrutiny was the process of scaling from animals to humans in determining risk. One regulatory agency, the Food and Drug Administration (FDA), uses a scaling factor based on the difference in body weight between rats and humans. The other major regulatory agency, the Environmental Protection Agency (EPA), scales up on the basis of surface area. On average, the two methods result in estimated risks that are a factor of ten apart.

For 15 years the two agencies have agreed to disagree on the discrepancy, says William Farland, director of EPA's health and environmental assessment office. There were good scientific arguments for both methods, he points out. But the FCCSET committee looked at the same data and came to a different conclusion. Neither method is obviously right, the panel said, so why not find a compromise? It recommended that both agencies adopt a scaling rule based on body weight to the three quarters power.

Because of a paucity of human epidemiological studies and no good way to convert from high animal doses to low human doses, EPA and FDA have been forced to rely on default assumptions and conservative models. As many as 40 assumptions are made in the absence of scientific data, leading to risk uncertainties of factors of ten. The result, says Paul Portney, vice president at Resources for the Future, is that "almost everybody has the uneasy feeling that we're setting risks too high".

And then there is economics. Regulatory costs associated with risk management are estimated to cost between \$60,000 and \$175,000 million a year — as much as 15 per cent of total current federal spending and 3 per cent of the GNP by 2000. For decades, government agencies have been prohibited from considering such costs when making regulation, by the so-called Delaney clause of the congressional Food, Drug, and Cosmetics Act, which requires FDA to prohibit food that has any amount of a substance that causes cancer in animals, regardless of any other considerations. Although Congress itself passed an exemption to the clause in the case of saccharin, where rat tests probably gave misleading indications of human risk, the rule still holds for pesticide residues in food.

Now, however, federal and independent analysts are questioning the goal of blindly eliminating all possible carcinogens, regardless of the side-effects of regulation. They argue that the total effect of regulation — not just the effects of the substance under review — is the critical issue.

Leading the wave of big-picture evangelists is John Graham, director of Harvard

University's Center for Risk Analysis. Graham believes that the costs of regulation may actually be killing more people than the actual chemicals the regulations are intended to control.

Graham's argument goes like this: Poverty is an established risk factor for illness and premature death. In a 1980 study, men in families with annual income less than \$5,600 have a mortality rate of 140 per 10,000 population, compared to a rate of 80 for men who made between \$17,000 and \$22,000 a year. Or, as Graham puts it, "wealthier is healthier".

Government regulations cause some economic drain, both to industry, where they raise the cost of manufacturing and producing goods, and on the store shelf, where those costs show up in higher prices. More expensive goods mean a lower standard of living for everyone, but the effect can be most pronounced for those on the edge of poverty.

Graham calculates that every \$5 million in regulatory costs results in one life lost to the risk factors associated with poverty. Thus, for instance, if it costs \$10 million to eliminate a potential carcinogen that can be expected to cause only one death a year, the cost of the regulation in human lives would outweigh its benefits.

Not surprisingly, the theory has attracted its share of critics, although few doubt the central premise. "I'm not sure what the relationship is between EPA regulations and personal income, whether it's causal or coincidental", says Adam Finkel of Resource for the Future. But he, too, argues in favour of more economics in risk assessment. "We need to take a harder look at what we mean by the costs of something versus what we mean by its risks. Right now the balance is about 98 to 2 in favour of risks."

On Graham's side are such long-time risk assessment critics as University of California-Berkeley biochemist Bruce Ames, a long-time critic of government risk assessment. Ames points out that pesticides lower the costs of fruits and vegetables, making them more available to the poor. "I don't believe that chemical residues have ever caused cancer, yet it is well established that not eating fruits and vegetables can increase cancer risk", he says.

Inside the government, this argument has also found a receptive audience. "We're starting to look at the total combined burden to society, both health risks and economics", says EPA's Farland. By assuming that potential toxins should be eliminated at all costs, regardless of the larger picture, government regulators worry that they may have lost sight of their original purpose — overall health.

Christopher Anderson

Correction: Car-eating bacteria

THE article on the biodegradation of motor cars in eastern Germany (*Nature* **350**, 453; 1991) unfortunately misspelled *Penicillium janthinellum* and misnamed the fungus a bacterium. □

SOLAR-TERRESTRIAL PHYSICS

Europe woos Japan

London & Tokyo

EUROPEAN and Japanese physicists are poised to overcome the usual reluctance of the world's three main scientific blocs — Europe, Japan and the United States — to collaborate with one another in 'big science' projects. Hampered by a shortfall in funding of almost £10 million, a European group plans to ask physicists at Nagoya University in Japan to work with them to build a large radar array in the Arctic Circle.

The collaboration would rescue the European solar-terrestrial physics community's most important project for the 1990s and save the Japanese from spending the money to build a separate system that would deliver basically the same data as the planned European radar array. But unless the European and Japanese groups can settle their differences over the design of the system, the partnership may never get off the ground.

Both the European Incoherent Scatter Radar (EISCAT) consortium, financed by funding bodies in six European countries, and a group at Nagoya University in Japan have been planning to build radar arrays on the island of Svalbard in order to study the interaction between the solar wind and the Earth's upper atmosphere and magnetic field. (Using incoherent scatter radar, physicists can study the movement, density and temperature of plasma in the ionosphere, several hundred kilometres above sea level.) At Svalbard, where it is dark at noon in winter, radar monitoring of the ionosphere can be combined with optical observations of auroras.

Originally, EISCAT planned to build a Polar Cap Radar consisting of three steerable radar dishes, at a cost of more than £20 million. The proposal was advanced most strongly by British physicists, but the recent financial difficulties of the Science and Engineering Research Council (SERC) have forced the British to delay their £4 million contribution for two years (see *Nature* **349**, 551; 14 February 1991). Even after this time, funding from SERC is not guaranteed.

At a meeting in Grenoble earlier this month, EISCAT council members from France, Germany, Sweden, Norway and Finland agreed reluctantly to finance the bulk of the project until SERC can afford to contribute. But even with the still-uncertain British contribution, EISCAT expects to have only £12 million to spend, as the world economic recession restricts spending on science in most countries, and the German contribution is further constrained by the cost of reunification.

To get around the funding problems, EISCAT would like to join forces with the Nagoya University group, says EISCAT council member Stan Cowley from Imperial College, London. But merging the two projects may prove difficult. The Japanese, led by Nobuo Matuura, propose to build a

phased array radar consisting of several thousand small radar transmitters arranged in a grid. The advantage of this system is that the radar beam can be steered around the sky electronically, as opposed to the mechanical steering of a radar dish. With a phased array radar, the beam can be redirected many times a second, whereas steerable dishes take several minutes to reposition mechanically. Matuura says he would like to collaborate with the Europeans, but only if EISCAT agrees to build a phased array radar.

Cowley agrees that the phased array design is technically superior, but he is worried about the cost. Cowley estimates that to meet EISCAT's specifications, a phased array radar would cost about £50 million. The European proposal requires a radar beam that can be steered over almost the whole of the sky above Svalbard. To do this, a phased array system (which can steer its beam through only a limited angle from the vertical) would have to be built on a tiltable platform.

Matuura replies that phased array systems are "not always as expensive as thought by the Europeans". A US-made phased array

radar could cost as little as \$20–\$30 million (£11–£17 million), he says. The cost of including a mechanical tilting system could be offset in part by building an array with fewer transmitters, Matuura says, putting a 50:50 collaboration with the Nagoya University group well within EISCAT's price range. Matuura expects his university to submit a formal request for funding for a collaboration with EISCAT to the Japanese Ministry of Education, Science and Culture next month.

If negotiations with Nagoya University fail, Cowley says that EISCAT plans to go ahead with a single dish system, rather than waiting for the economic climate to improve. The amount of science possible with a single dish would be much reduced — by about 50 per cent, according to Cowley. But he says it is important for the Polar Cap Radar to be working by the time the European Space Agency launches the Cluster mission in 1995. Cluster will consist of four space probes, flying in the solar wind. Data collected simultaneously from the Cluster probes and from the Polar Cap Radar would provide a unique opportunity to study the influence of the solar wind on the Earth's upper atmosphere.

Peter Aldhous & David Swinbanks

OTTO LILIENTHAL

Early flight pioneer commemorated

Munich

IN 1991 Germany celebrates the 100th anniversary of the first flight by aviation pioneer Otto Lilienthal (1848–96), whose scientific studies of the flight of birds led to the first reproducible glider flights.

Lilienthal, whose research was an inspiration to the Wright brothers, died in a glider accident in August 1896. The Wrights later made a gift of \$1,000 to Lilienthal's widow Agnes in gratitude for his contributions to aviation, which they had read about in the literature.



On a wing and a prayer.

thall gliders (a single- and a double-decker) as well as photographs and documentation with some English translations.

A poor student at school, Lilienthal became a successful engineer whose systematic aerodynamic studies of bird flight led him to realize the importance of a cambered (arched) wing. He published his results in 1889 in a book called *Bird Flight as the Basis of Aviation*.

Steven Dickman

Alvey lessons unheeded

London

THE British government has failed to heed many of the lessons taught by the Alvey information technology programme, the largest single new British research initiative of the 1980s, according to the author of a new report on Alvey. Many of the problems that afflicted the £350-million Alvey programme still plague government-sponsored research programmes, says Luke Georghiou from the University of Manchester.

In particular, Georghiou finds that the Alvey programme did not live up to its billing as a boost to the competitiveness of British industry. The findings are of special interest now as the British government is considering reforms to its £210 million LINK initiative — another programme of industrial/academic collaborative research that Georghiou says repeats many of Alvey's mistakes.

Georghiou and Ken Guy from the University of Sussex have just completed an independent evaluation of the Alvey programme, which ran from 1983 to 1988. Their report, commissioned by the Department of Trade and Industry and the Science and Engineering Research Council, concludes that Alvey was highly successful in strengthening the British information technology research community, but that it failed to fulfil its broader aim of improving the commercial performance of the British information technology industry.

Over the past few years, the British computer industry has continued to decline, and the report says that few of the companies that participated in Alvey projects seem likely to gain commercially from Alvey research.

Alvey brought together researchers from different companies and from academic institutions to work on so-called 'precompetitive' research. It concentrated on research into integrated circuits, software engineering, artificial intelligence and the interaction between humans and computers. In addition to £200 million of public money, Alvey projects consumed some £150 million provided by industry. The goal of the programme was that companies that collaborated in research projects should later go their separate ways and turn the resulting technology into marketable products. But the second competitive phase rarely materialized.

A number of factors were to blame. Alvey should have been accompanied by a training programme to address skills shortages in the British information technology industry, Georghiou says. He also believes that the focus on pre-competitive research was too narrow. In those cases where companies need to pool their expertise to work on a research project, they will often also need to collaborate in bringing the results of that research to market, Georghiou says. He believes that support for this 'near market' research and development is necessary if programmes such as Alvey are to yield

commercial results.

But since Alvey, the British government has actually reduced its support for near-market research. The Department of Trade and Industry's spending on industrial innovation, for example, dropped by some £50 million between 1987-88 and 1989-90. In recent months, the department has launched a small scheme to help fund the costs of developing commercial products in small companies (see *Nature*, 349, 556; 14 February 1991), but critics argue that this is too little, too late.

Brian Oakley, who headed Alvey's management team, agrees that the commercial exploitation of Alvey research has been disappointing, and blames a lack of incentive to invest in innovation. Over the past 20 years, he points out, the rate of interest on borrowed money in Japan has consistently been about one-third of that in Britain. This 'climate of expensive money' in Britain has kept companies from investing in research, product development and training, Oakley says.

Improving the climate for investment in innovation in Britain is a formidable task. But Georghiou says many of the difficulties that hampered Alvey have been retained in the government's current LINK programme, on which the government plans to spend £210 million of public money between 1988 and 1993. LINK also aims to unite industrial researchers and academics in precompetitive research, but it is not restricted to any particular industrial sector.

In some cases, Georghiou says, the rules governing the LINK programme are more perverse than those that hampered Alvey. The government provided 50 per cent of the research costs of companies involved in Alvey projects, and Georghiou believes that even more generous support was needed in order to encourage more companies to take part. But, for LINK, the government will pay only half the total cost of each collaborative project, including the funds for both industry and academic researchers. As academic collaborators are usually funded in full, this means that companies participating in LINK projects must usually pay most of their own research costs.

Another difficulty with Alvey, according to Georghiou, was the decision to negotiate intellectual property rights agreements before starting each project. The basic arrangement was that individual property rights would lie with the industrial partners in each project. But some projects were delayed by 18 months as the collaborators argued over the rate of the royalties that would be paid to university-based researchers in the event of any commercial returns. LINK has been subject to similar delays.

Oakley defends his decision to negotiate individual property rights agreements at the start of each project, saying that the negotiation of these agreements at a later stage might

have created further obstacles to commercial exploitation. Some of Oakley's former colleagues, however, agree with Georghiou. David Thomas, industrial liaison officer at Imperial College, London, and formerly an Alvey director, says the property rights issue "was an impossible problem".

The evaluation of Alvey and the criticisms of LINK are timely. The government is now considering its response to an independent review of LINK programme's management, commissioned because of the disappointingly slow take-up of LINK grants.

The LINK review, produced by the consultants Segal Quince Wicksteed, is to be published in the next few weeks, and its recommendations are thought to echo many of the criticisms voiced by Georghiou. The government may consider revising the rules governing individual property rights arrangements. But in the current harsh economic climate, a decision to increase the size of LINK grants made to the industrial collaborators in LINK projects is less likely, unless the number of new projects is to be reduced.

Peter Aldhous

ANTHROPOLOGY

Bones go home

London

THE University of Edinburgh is planning to return its collection of more than 300 Aorignal bones to Australia. The decision, announced earlier this month, is a victory for Aboriginal civil rights campaigners, who will take custody of the bones, but the move has disappointed the small international community of physical anthropologists, who used the collection to study the interrelationships between Aboriginal tribes. The bones will be returned in several months' time, once they have been catalogued in full.

Loring Brace, from the Museum of Anthropology at the University of Michigan, says the Edinburgh collection, amassed during the late nineteenth century, is one of the world's finest.

Unusually, the vast majority of material in the collection is identified according to its region of origin. This allowed researchers to study the relationships between Aboriginal tribes, and their rate of evolution. "There is no comparable collection" of Australian Aboriginal bones, says Brace.

Some research should still be possible, by taking measurements from casts and photographs of the bones, but the loss of the original material will rule out any future genetic studies, made possible only recently by the advent of new DNA amplification techniques.

The University of Edinburgh decided in principle earlier this year to return human remains from its collection on request — provided the remains were deemed to have cultural or religious significance to the group concerned.

Peter Aldhous

A universal Hamlet?

SIR — J. R. Smythies' suggestion that Wittgenstein was paranoid (*Nature* 350, 9; 1991) is intriguing and calls for comment.

It is generally known that before the worldwide acceptance of the DSM III criteria, the diagnosis of schizophrenia was used with quite different meanings by North American, European and Soviet psychiatrists, and that there are still no biological tests for schizophrenia. The situation today is well illustrated by the aphorism of Pascal: "When we do not know the truth of a thing it is of advantage that there should exist a common error."

In the wittgensteinian sense, the concept of schizophrenia could even be seen as a spurious attempt to grasp and classify human madness in scientific terms, and Smythies' letter is a warning of what may happen when people are privileged by virtue of their psychiatric training and identification to decide what is sound and scientific.

The following note by Wittgenstein demonstrates that he was deeply aware of the hazards inherent in his way of thinking, doubting and perceiving the world:

Ich sitze mit einem Philosophen im Garten; er sagt zu wiederholten Malen "Ich weiss, dass das ein Baum ist" wobei er auf einem Baum in unserer Nähe zeigt. Ein Dritter kommt daher und hört das, und ich sage ihm: "Dieser Mensch ist nicht verrückt: wir philosophieren nur".

[I am sitting with a philosopher in the garden; he says again and again "I know that that's a tree", pointing to a tree that is near us. Someone else arrives and hears this, and I tell him: "This fellow isn't insane. We are only doing philosophy."]

Thus the claim that the writings of Wittgenstein should be read as nonsense poetry lacking a scientific dimension is not surprising.

I have referred to Wittgenstein's studies on certainty in support of my theory that Shakespeare approached the mystery of madness in an experimental way by studying the genesis and consequences of a 'false' experience of truth (*Othello*, *Macbeth*), and what happens when our natural inclination to act according to the experience of truth is lost (*Hamlet*). I now propose that Wittgenstein, instead of being labelled as a schizophrenic, should rather be regarded as a universal Hamlet figure of our time.

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SIR — J. R. Smythies' letter¹ on Wittgenstein raises a number of questions: (1) If schizophrenia, a serious mental illness, is compatible with the achievement and international stature of Ludwig Wittenstein, should not the concept of mental 'illness' be re-defined? (2) Should scientific, philosophical

and literary texts, as well as people, be subject to medical certification? Should we question the validity of the kinetic theory of matter, because one of its originators, Ludwig Boltzmann, took his own life? (3) Is mental health conformity to a norm, or to an ideal? If the former is correct, is it abnormal to be extremely virtuous? (4) What is Smythies' professional opinion of such writers as Jonathan Swift, Edgar Allan Poe and Blaise Pascal (not to mention such extreme cases as those of Charles Baudelaire and Jean Jacques Rousseau)?

Incidentally, the metaphorical, or the highly suggestive, use of language, described as "schizophrenese" by Smythies, has met with a happier treatment by Pascal: "*Nous connaissons la vérité non seulement par la raison, mais encore par le coeur*"² — "We recognize truth not only through reason but also through the heart". Again, such an extreme rationalist philosopher as Spinoza acknowledges intuition as the highest form of knowledge ("*cognitio Dei intuitiva*"³ — "the intuitive knowledge of God").

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1. Smythies, J. R. *Nature* 350, 9 (1990).
2. Pascal, B. *Pensées*, p. 73 (Nouveaux Classiques Larousse, Paris, 1965).
3. Spinoza, B. *Ethica*, V. Prop. XXV (van Vloten & Land, The Hague, 1914).

Origin of AIDS

SIR — A. Karpas's letter on the "Origin and spread of AIDS" (*Nature* 348, 578; 1990) is surely premature in its conclusions. A combination of tribal customs involving African monkeys carrying HIV-like agents as the probable origin of AIDS in man is essentially speculative. However, the use of tissues from such animals for live human vaccine production is a fact and should be considered as a possible source of the virus. Such vaccines were also a post-Second-World-War development as was the introduction of syringes to Africa. The reference to "more than a dozen such cases" of herpesvirus simiae (B virus) infection in man, with no mention of millions of cases of SV40 transmission to man via poliovirus vaccine¹ prepared in monkey tissues, is misleading.

Tribal customs in Africa are age-old, yet this disease is obviously new even to this continent. That there are now many cases of the disease in Africa is not surprising in view of the poor health conditions in most developing societies, not only in Africa. There is also a marked increase in infection by the AIDS virus in Thailand and Brazil. The evidence pointing to Africa in the origin of AIDS is tenuous at best. "Continuous mutation" is unlikely to explain the wide diver-

gence between HIV-1 and HIV-2, their simultaneous appearance in man as disease agents and the continued existence in chimpanzees and other primates of closely related viruses. Even recombinants of simian viruses and endogenous human retroviruses cannot be ruled out. To single out Africa and its people and customs as essentially pivotal in this tragic pandemic is decidedly biased, and in view of the possible social and related repercussions one would urge extreme caution before embarking on such generalizations.

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1. Shah, K. & Nathanson, N. *Am. J. Epidemiol.* 103, 1–12 (1976).

Misuse of words

SIR — Although scientists use many words of Greek and Latin origin, they are often not familiar with the grammar of these ancient languages. Many words are also imitated and repeated without thinking about their real meaning. An example is the term 'recombinant'.

The Latin ending —ans, which became —ant in French and English, characterizes the present participle (active voice) and means —ing in English. Thus 'recombinant' means 'recombining'.

The use of this word is correct, for example, in 'recombinant technology'. But it is applied wrongly when speaking about the product of a recombination, for example a gene, after it has been recombined. Then it is not a 'recombinant gene' (recombining gene) but a 'recombined gene' (past participle, passive voice).

In Latin it would be '*recombinatus*'. Thus, if 'recombined' does not look learned enough, I would propose 'recombined': 'recombined gene' instead of 'recombinant gene' does not sound bad and would be logically and linguistically correct. What is now 'a recombinant' should properly be called 'a recombination product'. (Maybe someone can invent a better word.)

There is also an old word similarly used wrongly: the noun 'mutant'. It should properly be 'a mutatus', which admittedly is an ugly word. Is there a better one that is grammatically correct?

Unfortunately, the thoughtless mechanical misuse of the term 'recombinant' (and also 'mutant') is already so deeply ingrained that it will be almost impossible to eliminate it even partially from scientific jargon and papers. But by becoming aware of this attack on common sense and grammar, at least some readers with a linguistic feeling may in future try to avoid this frequent error.

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Margot O'Toole's record of events

The Baltimore saga continues. These excerpts from Dr Margot O'Toole's comments on the recent draft report by the US NIH's Office of Scientific Integrity contradict at several points last week's statement by Dr David Baltimore.

I THANK the Office of Scientific Integrity (OSI) staff and the scientific advisory panel for their months of dedicated work on what they have aptly termed "the tangled web of data and testimony". On the basis of clear and convincing arguments, the report correctly concludes that where actual experiments had been done, they do not support the challenged claims of the paper, and that other experiments were fabricated in an attempt to hide that fact.

Although I very much appreciate the draft report's praise for me, I believe my actions were nothing more than should be expected of any scientist. Unfortunately, attacks on my competence and motives began as soon as I raised questions; they continued for five years. This made what I did very costly and difficult for me. The draft report characterizes my actions as "heroic in many respects"; I would characterize my actions as conforming to the accepted standards of the scientific tradition, and the actions of the coauthors and university investigators as contrary to those standards and detrimental to the profession.

I am sure that as scientists come to know the history of this case, many will agree with my assessment.

In May 1986, I accidentally came across the laboratory records for the actual experiment on which the central claim of a paper published in April 1986 (*Cell* 45, 247; 1986) was based. It was obvious from these records that the experiment had not yielded the published results. This fact was, and remains, evident to many scientists who have examined the records. The draft report calls them "*prima facie*" evidence of problems. Dr McDevitt, a member of the original National Institutes of Health (NIH) panel, has stated that, based on these records, he would have "thrown the whole study out of the window" if Dr Imanishi-Kari had not presented him in 1988 with data that later proved to be fabricated.

I immediately attempted to determine if the discrepancies could be explained on the basis of additional data. I asked Dr Imanishi-Kari for the records for a repeat experiment, but she did not show them to me. A few days later, I therefore went for advice to Dr Huber, a more senior colleague.

She called together what has come to be known as the Wortis Committee. That committee and I gave Dr Imanishi-Kari the opportunity to explain the discrepancies, and we examined any and all additional data she presented in support of her published claims at a meeting on 23 May 1986. She

candidly admitted that certain crucial experiments described in the paper had not even been performed and that experiments that had been performed had not yielded the results claimed.

I left the meeting confident that the paper would be retracted. However Drs Huber and Wortis later telephoned me and told me no retraction would be submitted.

At the meeting, Dr Imanishi-Kari had said that all the discrepancies were the results of "mistakes, not dishonesty". Since I was in no position to know what her intent had been, I decided that I should give her the benefit of the doubt and not make a formal charge of fraud. But the central finding as published appeared exciting and novel, and I knew that other scientists would waste effort and time attempting, as I had for almost a year, to extend it. Therefore, I felt an obligation to seek a published correction.

I brought my evidence (a copy of 17 pages of data showing the published experiment) to officials at the Massachusetts Institute of Technology (MIT). Dr Eisen was appointed to investigate the matter. I showed him the records.

At his request, I prepared a memo on the scientific issues, which he gave to Drs Baltimore, Weaver and Imanishi-Kari, and he arranged for a meeting between these three coauthors, himself and me which took place on 16 June 1986.

As is now acknowledged by all parties, the only relevant data from Dr Imanishi-Kari's laboratory brought to this meeting were the 17 pages of data I brought. I have explained that these are the data the NIH draft report calls "*prima facie*" evidence of serious problems.

Dr Baltimore examined these data, the very data for the published experiment, and acknowledged that the published results could not be based upon them. Dr Imanishi-Kari again admitted that a large series of the published experiments had not even been performed, and that some that had been performed had not yielded the claimed results.

Some of Dr Weaver's data were also examined, revealing another inaccurate claim in the published paper. Since I had made no charge of fraud, a retraction at this time could have been made without disastrous consequences. But Dr Baltimore said he would oppose a retraction, adding that these kinds of inaccuracies were "not unusual". He told me that he would personally oppose any effort I made to get the paper corrected.

During the review of the paper that took

place at this meeting, the authors became aware of a printer's error, which was not scientifically misleading, in one of the figures. They submitted a correction (*Cell* 46; 4 July 1986) for this printer's error, having refused to correct the scientifically relevant inaccuracies that were by this time well known to Drs Weaver, Baltimore and Imanishi-Kari.

I dropped the matter after the 16 June 1986 meeting. But Mr Stewart and Dr Feder, who had been following it from the beginning, pursued it. In an effort to silence their persistent criticism of the paper, Dr Baltimore called for an official NIH investigation.

This left Dr Imanishi-Kari in a very vulnerable position. If she had not fabricated data in preparation for this investigation, the panel would have discovered that there were no data for the central claim of the paper. A retraction made as the result of an official NIH investigation might have had serious consequences, not just for Dr Imanishi-Kari, but also for those (Drs Baltimore, Eisen, Weaver, Huber, Wortis, Woodland) who claimed to have investigated my allegations and who had stated that they were unsubstantiated.

While this NIH investigation was progressing, the coauthors submitted a third publication (*Cell* 55, 541; 1988), acknowledging some fairly insignificant misstatements in the original paper. They stated that the misstatements were "not material alterations, and do not affect the conclusions of the paper, which remain appropriate and have been the basis of further studies".

The NIH panel reported in January 1989 that the paper contained "significant errors of misstatement and omission", but Dr Imanishi-Kari had presented this panel with additional data purporting to support the central finding. As the draft report makes clear, it is now known that this additional support was fabricated, something which had not yet come to light in 1988. I received a copy of the report in late 1988 before it was released, and I immediately informed NIH that Dr Imanishi-Kari had admitted that the experiments cited in the report had not been performed.

My statement was relayed to Dr Baltimore, and even with this statement in hand, he submitted the fabricated data as part of his fourth publication on the study (*Cell* 57, 515; 1989). This publication read, in part, "Nothing in this clarification affects the conclusions or interpretations in the original paper."

By early 1989, Drs Baltimore, Weaver,

Imanishi-Kari, Eisen, Wortis, Huber and Woodland had made statements to the NIH that contradicted the statements I had made. Relying on assurances from these seven people, the NIH issued a report stating that, despite the obvious problems with the experiments in the original paper, the central finding of the paper could stand.

Congressman Dingell, on the other hand, did not automatically assume that it was I who was untruthful. He called in the Secret Service and asked them to determine when the disputed laboratory records had been compiled. By 1989, the Secret Service had already determined that Dr Imanishi-Kari's notebook, which contained the purported supporting data, was not authentic with respect to time, and that some of the crucial records did not exist until after I had made allegations.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Margot O'Toole: "I lost my career because I would not go along with pressure to misrepresent my own results".

Before any of this evidence was made public, the Secret Service experts met with Dr Baltimore and told him of the evidence. In sworn testimony, Dr Baltimore characterized this presentation as "a charade of helpfulness . . . designed to terrify" him. Dr Baltimore continued his defence of the paper for another two years, saying in sworn testimony that "there is still nothing . . . that causes me to doubt the validity of the *Cell* paper".

Based on professional and personal assurances such as this from Dr Baltimore, many scientists rallied to defend the profession from what he characterized as an unwarranted intrusion by people seeking to "cripple American science".

When the Secret Service completed their analysis, the evidence proved that Dr Imanishi-Kari had fabricated data in order to deceive the investigative panels. The scientific advisory panel to OSI unanimously agreed that these data were fabricated. Only then, five years after he first learned of the problems, did Dr Baltimore retract the paper.

In summary, in 1986 I presented to Dr Baltimore *prima facie* evidence of serious discrepancies between experimental results and what he had published. Dr Imanishi-Kari admitted to him in my presence that some of the experiments had not been done, and that other experiments had not yielded the claimed results. Over the next five years, the authors refused to take any substantive

corrective action. Instead they repeatedly assured the scientific community that the findings reported in the original paper were solidly supported.

When the controversy became public in 1988, Dr Baltimore gave personal assurances to his colleagues, and called on them to help him defend against 'outsiders'. He and his coauthors published two explicit confirmations of the paper in *Cell*. The second of these confirmations included experiments now known to be fraudulent, and was published after Dr Baltimore had received my statement saying that the experiments had not been performed. He consistently and falsely maintained that my objections were no more than alternative interpretations of valid data. He defended Dr Imanishi-Kari as "the most intensely honest person" he knew.

Washington Times/Ruth Frenson

I consider it a disgrace that Drs Weaver and Baltimore did not immediately retract the paper in 1986 when they learned of the discrepancies between what was actually observed and what was published. Instead, it required four congressional hearings, months of Secret Service forensic work, two NIH investigations, years of effort on the part of the OSI staff and its advisory panel and a proof of actual data fabrication before the paper

was finally retracted. The cost to the public and the profession resulting from the stonewalling by the authors has been enormous. I wish also to make the following more specific comments on the draft report:

■ In her prepared statement to Congress in May 1989, Dr Imanishi-Kari stated: "All of the data were in the books which were available in the laboratory in May 1986". As documented in the draft report, that was untrue. The data shown to me at the 23 May 1986 Wortis Committee meeting and dated 1984 were not even written down until 19 May 1986. It is now established that the assembly of Dr Imanishi-Kari's notebook continued through the spring of 1988.

The draft report documents Dr Baltimore's remarkable admission that, at the time Dr Imanishi-Kari made this sworn statement, he already knew that the data were not in books in May 1986. Indeed, Dr Baltimore knew that the records consisted of, among other things, what his lawyer describes as "a lot of just loose paper" up until the time the authors were preparing for the 1988 investigation.

Given the knowledge Dr Baltimore now admits he already had at the time of the 1989 congressional hearing, there are only two conclusions he could have drawn: either (1) the data he saw on loose paper in 1988 were not the authentic support for the paper, or (2) Dr Imanishi-Kari's sworn statement that the data were already in books by May 1986 was untrue. But he nevertheless continued his

outspoken support for Dr Imanishi-Kari and the paper for another two years.

Moreover, at no point during the 1989 hearings nor, as I understand it, in his private meeting with the Secret Service, did Dr Baltimore reveal his prior knowledge that the records had been assembled in preparation for the 1988 investigations.

Even more troubling, this assembly had been done by Dr Imanishi-Kari on the advice of Dr Baltimore's lawyer, and with Dr Baltimore's knowledge and consent. And yet he did not reveal what he knew. Furthermore, according to the hearing transcript, after Dr Baltimore was briefed in private by the Secret Service, he was requested to meet the subcommittee staff to provide "such additional information as Dr Baltimore felt would be necessary or useful in connection with" the investigation.

When asked directly about the notebook during sworn testimony, Dr Baltimore still did not disclose what he knew. At the time of the 1989 hearing, the congressmen knew from Secret Service evidence that certain data dated 1984 had actually been written down in May 1986 (following my allegations). They did not yet know what Dr Baltimore already knew: the assembly of Dr Imanishi-Kari's notebook had continued for two years after I first made allegations. This exchange occurred between Representative Wyden and Dr Baltimore:

MR WYDEN: Why weren't the various panels that were looking into the 1984 data told it was not put together until 1986?

DR BALTIMORE: I don't think that's a question I can answer.

It now turns out that Dr Baltimore could have answered this question very easily. He could have said that he knew that the records had been assembled in preparation for the NIH investigation, and that he did not inform the NIH panel of this fact when they interviewed him. He could then have stated his reason for not informing them.

■ As I have explained, Dr Imanishi-Kari admitted at the 16 June 1986 meeting that certain experiments had not been done, and that others had not yielded the claimed results. Therefore I take issue with the statement in the draft report: "It may be understandable that Dr Baltimore initially failed to credit the questions raised . . .". It would be unfair to her, and would unfairly leave the burden entirely upon her, if I were to allow the report to stand without again emphasizing this point.

■ The draft report documents that a northern blot published by Dr Weaver was under-exposed and, as a result, the expression of the transgene (a *mu* gene) was not disclosed. The paper contained a specific claim that the transgene was not expressed in certain hybridomas. Low level production of the transgene could explain why the hybridomas were positive for the idiotype of the transgene. The fact that Dr Weaver privately observed a *mu* band while publicly claiming he did not is troubling. ▶

In his sworn testimony in 1989, Dr Weaver said: "Even if there were a low level expression of the transgene, it would not change the idiotype property of the cell." An elementary understanding of the experiments reveals that this statement is false. Note that after three years of defending the central claim, Dr Weaver made this statement, which can be explained in only one of two ways. Either Dr Weaver knew the statement to be untrue, or he does not understand the most fundamental facts about his own work. It seems inappropriate for the OSI to find either of these possibilities to be acceptable.

The draft report states that: "It appears that Dr Weaver was not alert to, nor concerned about, the significance of double producers [hybridomas which expressed both the transgene and endogenous immuno-

globulin]" I remind OSI that the existence and significance of double producers was discussed extensively at the 16 June 1986 meeting. Even if Dr Weaver had not understood the issue before, he certainly seemed to grasp it then.

■ The report does not address the issue of when the data published in the third correction (*Cell* 57, 515; 1989) were fabricated by Dr Imanishi-Kari. These data (June subcloning data) purportedly confirmed the claims of Table 2 of the original paper. The evidence shows that the fabrication did not occur until after the Wortis and Eisen reviews of May and June 1986. OSI determined that the data were not authentic through forensic tests, but I knew they were not authentic before any forensic tests were done.

Why is this? It is because I was told by Dr

Imanishi-Kari herself that the experiments had not been done. She stated this to Drs Huber and Wortis and to me. Moreover, she admitted to Drs Baltimore, Eisen and Weaver that, in preparing Table 2 for publication, she had relied completely on her prior expectation of what the results would be. She again admitted that the only experiments performed to characterize the cells listed in Table 2 were those recorded in the seventeen pages.

The Wortis Committee members claimed in May 1986 that they examined the data for the June 1985 subcloning. I have evidence (not quoted here) on two points attesting to the falsity of this claim.

■ During the 16 June 1986 meeting, Dr Imanishi-Kari admitted that Figure 1 in the *Cell* paper was not a truthful rendering of experimental results. Dr Baltimore told me

I note that the evidence of fabrication was so airtight that Drs McDevitt and Storb have finally agreed that Dr Imanishi-Kari's most important records are fabricated. They dissent from other portions of the draft report, but they do not explain the basis for their disagreement.

Because of the serious nature of the findings in this case, it is of the utmost importance that scientists know that all the findings of the draft report are solidly supported. The minority opinion states that, for some of the findings, "alternative scenarios are sufficiently plausible . . .".

This statement cannot be evaluated because Drs McDevitt and Storb do not describe a single alternative scenario that could explain away any of the evidence compiled by OSI. I therefore consider it imperative that the minority opinion be amended to include the scenarios that Drs McDevitt and Storb believe are plausible explanations. This would give the reader a basis for judging whether, as the minority members assert, their "alternative scenarios are sufficiently plausible. . .". Drs Storb and McDevitt should provide this information.

One possible scenario that could be easily evaluated for plausibility would be one which explains how the notations 3/20/85, 3/21/85 and 3/22/85 on the right hand corner of pages do not refer to dates. Of course the scenario would have to explain: (a) why Dr Imanishi-Kari initially claimed that the experiments depicted on the pages with these notations were done in March 1985; (b) why she now claims that she does not know the meaning of the 3/20/85 notation; (c) why pages with these notations are sequential, and preceded by pages with 2/7/85 notation and followed by pages with 5/22/85 notation in the upper right-hand corner. This entire series of experiments recorded with these notations is integral to the authors' defence against my challenge.

The minority report states "Unfortunately, the panel was not given access to the ink chromatograms . . ." used in char-

Questions for Drs McDevitt and Storb

acterizing the different inks used by different gamma counters at different times. I state for the record the 'scenario' that would have to be invoked to explain the evidence with or without access to ink chromatograms.

Nearly every time that Dr Imanishi-Kari used the gamma counter, she would have had to have removed both the roll of paper and the ribbon, and replaced them with a different paper and ribbon. Sometimes she would even have to have changed to a third ribbon while her samples were being counted. When finished, she would have had to have reinstalled the original roll and ribbon, so that the tapes of those who used the counter before her would match, in both paper and type density, the tapes of those who used the counter after her.

Even this preposterous explanation does not take into account what Dr Imanishi-Kari actually said when asked about the tape anomalies. She made no mention of having performed this extremely bizarre and inexplicable task. Instead she said that the tapes may have come from other counters, an explanation now known to be untrue.

The 'frequent-switching-of-ribbon-and-paper' scenario, however, does not explain the evidence, even if one ignores or discounts the ink chromatograms and Dr Imanishi-Kari's own statements.

What scenario do Drs Storb and McDevitt propose to explain the counter register numbers? One set of these numbers establishes December 1985 as the true date of the tapes 'coded' with the handwritten notation 3/21/85. Since the manuscript was being circulated in the fall of 1985, and was submitted for publication in December 1985, tapes generated in December cannot be cited as justification for the published claims.

It must be difficult for those who have not been involved in the many investigations of this case to comprehend why Drs Storb and McDevitt are still invoking 'sce-

narios' that Dr Imanishi-Kari herself has never claimed actually happened. Therefore I point out that, during my interactions with Drs McDevitt and Storb, OSI staff members explained to me that both were functioning as advocates for Dr Imanishi-Kari's position.

The advocacy role described by the OSI staff is not disclosed either in the OSI draft or in the minority opinion. For instance, I was told that I should respond to the imaginary scenarios Dr McDevitt presented because it was his role to think up any possible theory to justify dismissing my evidence. Years have passed while each one of these imaginary scenarios was being proved impossible. Now the minority opinion again invokes imaginary scenarios, but this time without even stating what they are. Hence these new scenarios cannot even be analysed to determine if they are consistent with the body of evidence.

Imaginary scenarios considered and disproved over the years include, but are not limited to: (a) all of the inaccuracies in the paper were due to Dr Imanishi-Kari's English; (b) Dr Reis (who is from Brazil) could not understand the Portuguese of Dr Imanishi-Kari (who is also from Brazil); (c) Dr Imanishi-Kari used many other gamma counters, all of which matched precisely the print format of the gamma counter in her own laboratory; (d) Dr Imanishi-Kari's gamma counter tapes were generated by some machine that was not even at MIT; (e) Dr Imanishi-Kari could keep track of the properties of many like-named hybridomas without maintaining a laboratory notebook; (f) Dr Imanishi-Kari selected for publication her very worst and most anomalous data, and saved her best data for the people investigating the anomalies in the published data.

I choose the above examples of scenarios proposed to me mostly because their plausibility can be evaluated without any

he would deal with this matter in private. My statements on this matter are corroborated by Dr Baltimore's 6 September 1986 letter to Dr Eisen, in which he clearly stated his knowledge of this misrepresentation which I had brought to his attention three months earlier. The letter further states that Dr Imanishi-Kari was by then admitting that she had "known all along" that the results had not been obtained. In this letter, he again stated that he would not issue a correction for the general community, but added that he would be candid with scientists in personal contact with him.

In congressional testimony, both Drs Eisen and Baltimore maintained that the letter was the result of a misunderstanding due to Dr Imanishi-Kari's poor English. Both failed to mention that, three months before the letter was written, they had been

knowledge of the scientific issues.

The minority opinion dismissed the statistical analyses as 'untested'. They object to the use of statistics "particularly in establishing proof of fraud".

I point out that the statistical evidence in this case is used only to corroborate findings solidly supported by other evidence.

It is untrue to say that the use of statistical analyses is untested in investigations of fraud. Moreover, their use in science is routine, unchallenged as a method in thousands of studies. Studies that do not include relevant statistical analyses are likely to be rejected on this basis alone.

In rejecting the statistical analyses, Drs Storb and McDevitt again do not describe the basis for their objections, so that their opinion cannot be evaluated. Specific expertise is a definite prerequisite for evaluating disagreements pertaining to statistical analyses. I do not personally have this expertise, but once Drs Storb and McDevitt explain their objections to the analyses done by an acknowledged authority in biostatistics, scientists in general can evaluate the relative merits of the arguments.

Drs Storb and McDevitt dissent from the conclusions in Section V, but they do not explain which conclusion they reject and for what reasons. Thus once again their report cannot be evaluated and answered. In an interview with *Science*, it appeared that Dr McDevitt was dissenting from portion C of Section V, the part relating to Dr Baltimore.

The minority opinion makes it clear that both dissenters agree with the basic finding that Dr Imanishi-Kari fabricated the evidence she presented to them, so it is unclear why they dissented from this portion of the report. They give no explanation for dissenting from the portions of Section V that deal with Dr Weaver and myself, and therefore I cannot comment further. I request that the minority opinion be amended to include the reasons why Drs McDevitt and Storb rejected each of the Section V determinations.

informed of this same fact by me, both orally and in writing — and English is my first language.

Dr Baltimore said in testimony that the reagent worked some of the time. Now the draft report finds that the data submitted to the NIH in support of this explanation are fabricated. The explanation itself had not yet been devised in June 1986 nor, it appears, in September when Dr Baltimore wrote the letter.

■ It has long been established that, contrary to the claim of the paper, no test for IgG was done on the Table 2 hybridomas. The authors eventually 'corrected' this false claim by replacing it with another false claim. They said the claimed tests had instead been run on the Table 3 hybridomas. It was fortunate for me that the actual results for the Table 3 hybridomas had already been reported in Dr Eisen's grant application and lay buried among NIH documents.

Without this evidence, it is conceivable that OSI would not have been able to unravel this part of the "tangled web".

■ There is another basic fallacy inherent in Dr Imanishi-Kari's defence that is not addressed in the draft report. Dr Storb told me that she was discounting all of the evidence showing that Dr Imanishi-Kari's notebook had been written up after my challenge because, after Dr Imanishi-Kari was confronted with proof of this, she gave a new explanation. She went from claiming that the notebooks were written up in a basically contemporaneous and timely fashion to admitting that they were not written up until after I had raised questions.

Drs Storb and McDevitt, in particular, accepted this flip-flopped story as justification for dismissing large bodies of forensic evidence. Even the draft report goes so far as to say that the fact that the notebook "did not exist as an entity prior to the first NIH investigation . . . would not in itself constitute a problem".

I point out a plain and simple reality. Scientists must keep records of experiments because they need the records to proceed from one step to the next. In this case in particular, there was a claim of a complicated analysis on a large series of like-named hybridomas over a long period of time. During this time, grant applications and the *Cell* paper were submitted. If the data were authentic, Dr Imanishi-Kari would have had to have recorded them as the results were being obtained. The fact that the records shown to me in 1986 were not created until I asked to see them is, in and of itself, strong evidence of fraud.

■ I am pleased that the draft report included statements that the central and challenged claim of the paper has not been replicated. Indeed the results have never been obtained, not even once. I take this opportunity to state my own stand on the subject of replication: failure to replicate a report does not demonstrate that fraud has been perpetrated. Therefore, I did not assume fraud when I

observed that the idiosyncratic expression and transgene expression always seemed to go together.

It was not until I found the data for the actual published experiment that I knew that the result had never been obtained, and even then I accepted the "error not fraud" possibility, remote though it seemed.

The report states that I lost my job as a result of raising questions. This is essentially true, but the facts are a little complicated. It is more accurate to say that I lost my career because I would not go along with Dr Imanishi-Kari's pressure to misrepresent my own results.

I informed Dr Eisen of this pressure, and he, as director of my training programme, had a direct responsibility to help me. He failed in this and other responsibilities to me, and to the NIH with respect to me. It is also true that Dr Mary Rowe, an assistant to the president of MIT, told me that MIT would ensure that a fair and thorough investigation was conducted and that I would not be left unemployed as a result of raising legitimate questions.

After the 16 June 1986 meeting, both Drs Eisen and Rowe refused my urgent request for help and redress. When I told Dr Rowe I had acted with her assurances of fairness and thoroughness, she told me that the matter was "in the hands of God". I was left unemployed, and subjected to five years of slander and libel from Drs Baltimore, Eisen and Imanishi-Kari. Also, because of the cover-up at MIT, Drs Huber and Wortis were able to continue with their slander and libel of me. I bring this up to document what can happen to a scientist merely for adhering to the professed standards of the profession.

It surprises me that the principle which guided my actions is not a universally accepted one among scientists. For instance, there is an eminent scientist who has been, and continues to be, a most outspoken critic of what I did. Yet he was invited to deliver an important public seminar on scientific misconduct last month.

This shows that this scientist is regarded, at least by some, as an expert on the ethical issues facing scientists.

He and others have stated that, in this case, the protection of careers must take precedence over scientific accuracy. This closely echoes what Drs Wortis and Huber said to me in May 1986, what Dr Baltimore wrote to Dr Eisen in September 1986 and what the reviewers of the Stewart and Feder manuscript said. It also echoes what many others report happened when they brought forward evidence of scientific misconduct. I therefore sadly conclude that the attitude that scientific careers are much more important than science has become common among scientists.

However, I have come to see this case as one which could help change this attitude. This is why it is important for scientists to understand what happened.

Margot O'Toole

Oddballs from the Cambrian start to get even

Stefan Bengtson

A NEW onychophoran-like fossil, described by Ramsköld and Hou on page 225 of this issue¹, makes the Cambrian bestiary look a lot less bizarre than it used to. Onychophorans may appear to be odd, a cross between a centipede and Michelin Man, but only because they are unfamiliar to most of us (they now mainly live among damp leaves and rotting logs in the Southern Hemisphere). Ramsköld and Hou's paper suggests that they were more widespread and common than their present-day terrestrial confinement indicates, and that they may have been a notable component of the earliest metazoan biotas.

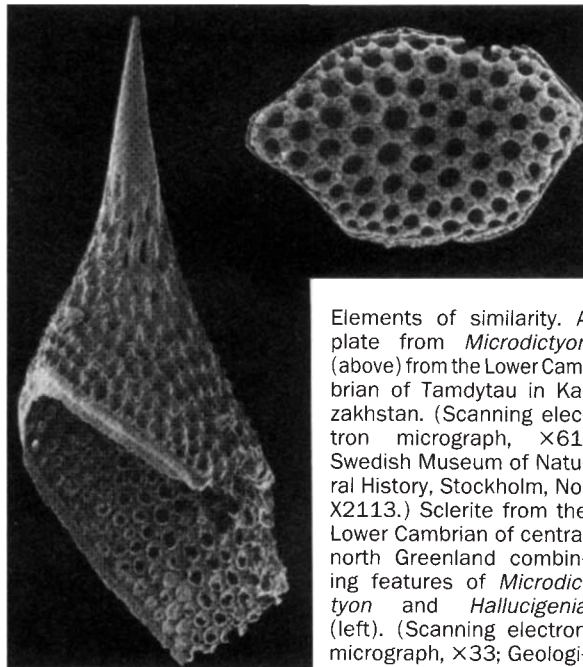
There have already been indications that such animals existed in the Cambrian: certain soft-bodied fossils resemble onychophorans and the microscopic tardigrades ('water bears')²⁻⁴. The fossil now reported from the Lower Cambrian Chengjiang fauna of south China, dating to 520–530 million years ago, further strengthens these comparisons. But Ramsköld and Hou's contribution goes further, in that it proves that enigmatic fossils such as *Hallucigenia* and *Microdictyon* also belong to the same clade and that a number of widely occurring sclerites (skeletal elements) of unknown affinity are parts of onychophoran-like animals. This has consequences for more general questions, such as the nature of the earliest metazoan assemblages that appeared in the 'Cambrian explosion' and the significance of the great number of problematic fossils from this time.

Described in 1977 (ref. 5), *Hallucigenia* has been a prize 'problematicum' of the Cambrian. It was reconstructed as an animal that walked on seven pairs of stiff stilts and that had a single row of seven tentacles along the back. Although some palaeontologists have aired doubts regarding the reconstruction, most have accepted *Hallucigenia* as a unique Cambrian organism that falls outside the traditional phylum divisions⁶.

The net-like sclerites of *Microdictyon* (see figure) are widely distributed in the Cambrian⁷. When the first fossils of complete specimens of the animal were discovered at Chengjiang⁸, palaeontologists were surprised to find the phosphatic sclerites spaced along the flanks of an appendiculate worm-like animal (see the front cover of this issue). The advantage of this arrangement was not obvious, but an intriguing possibility presented itself: *Microdictyon* bore a strong resemblance to *Hallucigenia*, provided that one of the animals was turned upside down so that the 'appendages' came to occupy the same position as the 'tentacles' of *Hallucigenia*. The 'stilts' of the latter would be homologous to the

sclerites of *Microdictyon*.

But which way was up? *Hallucigenia*'s tentacles had been rather definitely described as unpaired, and although the published reconstruction of *Microdictyon* sported paired appendages⁸, the specimens appeared to show only a single row. An



No. MGUH 20670, sample 315108, collected by J. S. Peel.)

animal tottering along on a single row of tentacles seemed scarcely less hallucinatory than one perched on stilts, but the stilt-walking model just didn't work for the low plates of *Microdictyon*. Also, the suggestion that *Hallucigenia* was some detached organ of a larger animal⁹ lost its attraction now that the complete *Microdictyon*, having no more parts than *Hallucigenia*, was known.

The new Chinese fossil solves the puzzle. It clearly has characters in common with *Microdictyon* and *Hallucigenia* as well as onychophorans. The appendages are paired, and Ramsköld and Hou convincingly argue that the unpaired appearance of the appendages in *Microdictyon* and *Hallucigenia* is due to the mechanics of burial in sediment. A timely confirmation of the close affinity between *Microdictyon* and *Hallucigenia* also comes with the recent discovery of sclerites that combine the exquisite net-like structure of *Microdictyon* plates with the thorny shape of *Hallucigenia* 'stilts' (see figure).

The abundance of problematic fossils in the Cambrian is now widely seen to reflect unpruned diversity after the first major metazoan radiation⁶. The practice of 'shoe-

horning' in palaeontology (to classify, more or less routinely, problematic fossils with the least dissimilar living phylum) tended to prune phylogenetic bushes into phylogenetic trees, and many Cambrian palaeontologists have therefore adopted another strategy — analysis of the relationships within the groups of Cambrian animals, without forced homologies with characters in living animals, to build up the taxonomy from the implied phylogeny rather than shoe-horning taxa into a system that accepts only living phyla. If the characters are insufficient, as they frequently are, the principle is to keep the tag 'problematicum' until better data (or ideas) are available.

This approach may avoid the bias against extinct taxa. But there is a difficulty with it, one vividly demonstrated by the new discovery. Without known living relatives, there are few constraints on the reconstruction of a fossil. The earlier attempts to understand *Hallucigenia* and *Microdictyon* without recourse to assumptions about phyletic affinity^{5,7} failed as miserably as those that had made the wrong such assumption (the spines of *Hallucigenia* were originally interpreted as annelid setae¹⁰, and the sclerites of *Microdictyon* have been classified as radiolarian tests¹¹).

Whether we like it or not, fossils that belong to living phyla will always be easier to understand than those that don't. Extinction blindfolds

the palaeontologist, whose job after all is to study the extinct. Shall we then never understand life forms that do not have living relatives presenting similar anatomy? No doubt we shall, but the revelation that some of the most 'bizarre' Cambrian creatures now look more like onychophorans with shoulder pads is a healthy reminder that any fossil — whether an isolated sclerite, a preserved soft body or something else — presents but a fraction of its characters for inspection.

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3. Dzik, J. & Krumbiegel, G. *Lethaia* **22**, 169–181 (1989).
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5. Conway Morris, S. *Palaeontology* **20**, 623–640 (1977).
6. Gould, S.J. *Wonderful Life: The Burgess Shale and the Nature of History* 1–347 (Norton, New York, 1989).
7. Bengtson, S., Matthew, S.C. & Missarzhevsky, V.V. in *Problematic Fossil Taxa* (eds Hoffman, A. & Nitecki, M.H.) 97–115 (Oxford Univ. Press, New York, 1986).
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9. Collins, D. *Earth Sci. Hist.* **9**, 163–165 (1990).
10. Walcott, C.D. *Smithson. misc. Coll.* **57**, 261–364 (1911).
11. Hao, Y. & Shu D. *Geoscience* **3/4**, 301–310 (1987).
12. Conway Morris, S. & Peel, J. S. *Nature* **345**, 802–805 (1990).
13. Bengtson, S. *Nature* **345**, 765–766 (1990).

As did Conway Morris and Peel's description of a complete Cambrian halkieriid a year ago^{12,13}, Ramsköld and Hou's discovery opens up broad avenues of research into various Cambrian fossils, the origins and functions of mineralized cuticles, and the phylogeny of early metazoan IMMUNOSUPPRESSION

Binding by design

Dagmar Ringe

The reports in this issue by Moore *et al.*¹ (on page 248) and in *Science* by Michnick *et al.*² and Van Duyne *et al.*³, of the structure of a small intracellular protein called FKBP, could help in the design of new immunosuppressive drugs, as well as in the unravelling of the mechanisms of cytoplasmic signalling in T lymphocytes.

The immunosuppressive drugs FK506, which binds to FKBP, and cyclosporin A, which binds to cyclophilin, are structurally unrelated but seem to have a similar mechanism of action involving this initial binding. Both cyclosporin A and FKBP are intracellular proteins with peptidyl-prolyl *cis-trans* isomerase activity which could either be fortuitous or be the essence of the immunosuppressive action. Rarely have such small proteins received so much attention — the consequence is a plague of publications about them; more collaboration and less competition in this field would be a damn good thing.

Cyclosporin A and FK506 have generated such interest because they are highly effective in preventing organ-transplant rejection and in the treatment of auto-immune disorders⁴. The mechanisms of immunosuppression by these compounds are so far unknown, although both inhibit similar steps in the activation of T-helper lymphocytes⁵. A step forward in determining the actions of these compounds was the identification and isolation of the proteins to which they bind with high affinity. These 'immunophilins', cyclophilin and FKBP, are ubiquitous cytosolic proteins with no apparent sequence similarity⁶, and no known function. The discovery that they have peptidyl-prolyl *cis-trans* isomerase (rotamase) activity has suggested a mechanism whereby the immunosuppressive effect is achieved^{7,8}.

Isomerization of proline from *cis* to *trans* seems to be the rate-limiting step in the folding of many proteins *in vitro*⁹. Both cyclophilin and FKBP catalyse this reaction, albeit poorly¹⁰. Both enzymatic activities are inhibited by the respective ligands, cyclosporin A and FK506, but with no cross-reactivity^{7,8,11,12}. Therefore, cyclosporin A and FK506 could cause immunosuppression by inhibiting the rotamase activity. There is kinetic evidence for a mechanism of isomerization based on distortion of the susceptible peptide bond¹⁰. Inhibition could occur as a result of the recognition by the immunophil-

lineages. And friends of the onychophorans will be delighted at the rise to prominence of this little-appreciated group. □

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ins of a conformation in part of cyclosporin A or FK506 that mimics that of a transition state in the isomerization of *cis* to *trans* proline. Analysis by NMR of the conformation of FK506 bound to FKBP suggests that the drug stabilizes and recognizes a twisted peptidyl-prolyl amide bond of the ligand¹³, a mechanism that also seems to operate in the rotamase activity of cyclophilin¹¹.

How does this catalysed activity relate to immunosuppression? The complex series of events which result in the T-cell activation cascade require several days to occur¹⁴. Cyclosporin A and FK506 seem to act within the initial stages of this process. Stimulation of the T-cell receptor (TCR) by foreign antigen presented on the surface of an antigen-presenting cell activates a TCR-mediated signal transmission pathway. The signal is transduced through the cytoplasm by an as yet unknown mechanism and leads to the activation of specific transcriptional activators, such as the nuclear factor of activated T cells, NF-AT⁵. These factors help regulate transcription of T-cell-activation genes. Translation of the resultant message is followed by secretion of interleukin-2. Cyclosporin A and FK506 are potent inhibitors of this pathway, the drug-immunophilin complex being implicated as the inhibitor of this T-cell activation.

One mechanism by which this biological effect might be explained is that immunophilins have a more general function such as aiding protein folding by acting as foldases. Folding of small proteins can occur with half-times as short as 0.1 seconds. Larger proteins may require minutes with an average first-order rate constant for refolding of 1 s^{-1} (ref. 15). The rate of *cis-trans* isomerization of a model peptide at 37 °C is 0.15 s^{-1} , so if proline isomerization is the slow step in the folding of a protein in the transduction pathway, the rotamase activity of the immunophilins could control timing¹⁶. But there is some concern that immunophilins cannot effect rate acceleration of any real physiological importance unless present in large amounts or unless the acceleration needed for the critical isomerization is minimal.

A possible resolution of this paradox has emerged from a kinetic study designed to identify a specific substrate for the enzymes¹⁰. The results support the idea that lymphocytes contain specific substrates or unique forms of rotamases that can account

for the selective sensitivity of these cells to cyclosporin A and FK506. The results also indicate that selective inhibitors can be designed for cyclophilin, FKBP and other members of this family of enzymes. Although inhibition of the isomerase activity does not appear to be sufficient to prevent lymphocyte activation¹⁷, it is widely believed that the immunosuppressive activity of both inhibitors is mediated through the respective binding proteins¹⁸.

To design cyclosporin A and FK506 analogues with clinically useful immunosuppressant activities it is essential to know the structures of cyclosporin A, FK506, cyclophilin and FKBP. Specifically, a race is on to identify the portions of cyclosporin A and FK506 that bind to the protein, especially in the conformations that are most productive for the observed effect. Structures of cyclosporin A^{19,20} and FK506²¹ in crystals and solution have been used to guide the synthesis of analogues. To gain more direct insight into the molecular details of the function of cyclosporin A, the structure of the drug bound to cyclophilin has been determined by NMR using heteronuclear editing techniques^{22,23}. The alternative approach to using the structure of the ligand as a model for analogue design is to model new and possibly radically different inhibitors based on the structure of the binding site of the receptor. But so far no structure has been determined for any immunophilin, only for the drugs themselves either in isolation or when bound.

The structures of FKBP in solution^{1,2} and the FKBP/FK506 complex in the crystal³ are now reported, of which the complex is potentially the most important. The secondary and tertiary structures of the three independently solved structures are similar, and no obvious similarity between them and those of any other proteins of known function is reported. Given the small size and simple architecture of FKBP, structure comparison mavens will no doubt find numerous topological relationships. The solution structures agree in that a large hydrophobic cleft in the vicinity of residues 30–55 of FKBP comprises the binding site for FK506, an interpretation that is confirmed by the crystal structure of the complex. This location is consistent with the results of spectroscopic and mutagenesis studies that implicate tryptophan 59 in the recognition of the drug²⁴. The most striking result is the difference in conformation between free and bound FK506, a difference which may help to explain the rotamase activity exhibited by these proteins.

What use is the structure of FKBP without FK506 bound? So far, the results say little about the mechanism of rotamase action, and there is no obvious additional function to be deduced from the structure itself, as might have been the case had the protein resembled the structure of, say, one of the interleukins of some other immune modulator. But, knowledge of the structure of the target, especially

of the complex of the target with an important drug, should lead, through model-building studies, to the design of better drugs. This premise needs a closer look.

Model building of both the binding of drugs to proteins and the structure of homologous proteins has become fashionable, partly because of the explosion of sequence information and partly because the computational and graphical tools now exist to make the process relatively easy. Recent successes with the design of inhibitors for the protease of the human immunodeficiency virus from homology-based models of its structure²⁵ would seem to indicate that if the actual structure of the target protein is known, as is now the case for FKBP, one should be able to model and design from scratch first- and second-generation inhibitors. The discovery that the drug haloperidol also inhibits the protease of the human immunodeficiency virus was made by searching a database of the structures of small molecules for compounds with the same shape as the active site of the target enzyme²⁶. Can we now expect to design better immunosuppressives?

Consideration of other protein-inhibitor complexes makes one less sanguine. Inhibitors do not always bind in their minimum-energy conformation²⁷, so the search of structure databases may overlook many important compounds. And, of course, the structure of the target protein itself could change greatly when the drug binds. Nor is it obvious how precisely a target structure must be known to guide 'rational' drug design. The best NMR structures can have imprecision of angstroms in the position of their

mobile atoms. Is that tolerable, given the plasticity of protein structure? Perhaps, although there is no supporting evidence on this point.

Finally, the mistake most often made in modelling is to assume that closely related compounds will bind nearly identically. But structural data have shown that even a small change in ligand structure can completely change the preferred mode of binding of drugs to proteins, which is governed by many weak interactions. Only knowing the struc-

GLACIAL CLIMATE

No change down under

W. H. Berger

THE discovery, a decade ago, that the carbon dioxide content of the atmosphere during glacial periods was considerably lower than it is now^{1,2} produced a flurry of speculative papers on the role of plankton in regulating the ocean's, and so the atmosphere's, CO₂. For example, there was a proposal that the amount of phosphate nutrients in the global ocean varied as a result of sea-level fluctuation³. Many of the early models, and a number since⁴, have focused on the Antarctic Ocean as a likely place for great changes in biotic productivity. Now it seems, from the results reported by Mortlock *et al.* on page 220 of this issue⁵, that this focus may have been misplaced: these palaeoceanographers find no evidence of elevated productivity in the Antarctic during glacial periods.

These results are disconcerting, because they all but demolish one potential regulatory mechanism for atmospheric CO₂, one which has been considered quite powerful by many geochemists and palaeoceanographers. Without this mechanism, the observed⁶ variations in atmospheric CO₂ become yet more enigmatic.

Oceanographers look to the Antarctic Ocean in modelling these changes because there are vast reserves of poorly used nutrients in the uppermost 'photic' zone of the sea, where light-harvesting plankton reside. In contrast, most other large open-ocean regions have only just sufficient nutrients to support their biotic communities, so that any additional productivity would require substantial influences of nutrients from outside. Some change in conditions to allow nutrients to be used more fully in the Antarctic, the conventional argument goes, would permit more carbon to be pumped out of shallow waters to deeper waters, reducing the concentration of CO₂ dissolved in the surface waters which could then absorb CO₂ from the atmosphere.

The lack of a mechanism for increasing Antarctic productivity during glacial periods has been something of an irritant. Hence the interest in John Martin's proposal⁷ — that an increased supply of iron could be the necessary stimulus. Martin noted that ice deposited in Antarctica during the ice ages contains

more dust than that deposited now⁸. He argues that this wind-borne dust would have carried additional iron to the surface waters of the surrounding ocean, causing a bloom of phytoplankton. His hypothesis of iron-limited productivity was challenged by several prominent biological oceanographers at a recent meeting (see Philippa Lloyds' News and Views report⁹). They emphasized the importance of grazing pressures and lack of sunlight in maintaining high nutrient contents in the surface waters of the Antarctic. Martin responded by pointing to results of experiments that he and his coworkers performed¹⁰, which (he argues) confirm his views that iron is the limiting factor.

Now his "iron bullet" solution to the enigma of glacial carbon dioxide faces a different challenge, one based on palaeoceanographic reconstruction¹: if the postulated change in productivity did not happen, the need to explain it vanishes. Mortlock *et al.* look at three different proxies for palaeoproductivity: the accumulation rates of opal, the Ge/Si ratio in opaline skeletons in Antarctic sediments, and the contents of isotopic ¹³C in foraminiferal carbonate. The first of these proxies is straightforward, the other two rest on more complex arguments.

Accumulation rates of opal reflect the fallout from diatom production, minus dissolution on the sea floor. It seems that these rates actually were higher during glacial periods than now — north of the polar front zone (which is centred near 50° S). But south of this zone, they were considerably lower presumably because diatoms fared less well owing to a greatly expanded cover of ice. This overall difference in the sense of change resulted in the well-known northward displacement of the Antarctic belt of siliceous ooze during glacial times¹¹. Considering the balance between increases and decreases in silica accumulation, Mortlock *et al.* find that opal burial in the entire Southern Ocean south of 40° S was more likely to have been reduced, rather than increased, during the last glacial period.

Taken at face value, this result would demolish the theory of CO₂ control by Antarctic productivity, along with Martin's iron-

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CHROMOSOME KINETICS

Movement on two fronts

Richard Vallee

bullet solution. But it cannot be assumed that the accumulation of opal on the sea floor has a one-to-one relationship with carbon pumping. Although results from sinking detritus caught in traps show a close relationship between organic carbon and silica settling through the water column¹², the same relationship need not be valid at the sea floor or within glacial periods.

For example, there is evidence from the western equatorial Pacific that the rate of burial of diatoms decreased during the last several glacial periods (C. B. Lange, personal communication), at the very time that the supply of organic carbon to the sea floor increased (as seen in deep-water productivity: J. C. Herguera, personal communication). Possibly, dissolved silicate was generally in short supply during glacial times, so that opal deposition deteriorated as a monitor of productivity. Perhaps silicate was pumped out of the glacial ocean within continental margins, or less of it was supplied from land, owing to decreased chemical weathering on cold and dry continents.

Mortlock *et al.* reinforce their interpretation of the silica record with their analyses of Ge/Si ratios in the opal, and of ¹³C/¹²C ratios in planktonic foraminifers, *Neoglobobulimina pachyderma*. But although the first argument (that increased diatom productivity depletes Ge more strongly than Si) is attractive, it rests on the assumption of a constant Ge/Si ratio within the subsurface waters. And the stable carbon isotopes of *N. pachyderma* are difficult to interpret because this species lives in subsurface waters during much of its life cycle.

In sum, Mortlock *et al.* stage an impressive attack on the problem of Antarctic productivity changes; it should severely damage conventional belief in Antarctic modulation of atmospheric carbon dioxide. Yet, the basic question is not resolved. The Antarctic has not been eliminated from contention as co-regulator of CO₂, and Martin's iron hypothesis has not been discredited. In any case, Martin's hypothesis is not strictly tied to the Antarctic, as it could be applied elsewhere, for example in equatorial upwelling regions. □

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THE mechanism of chromosome movement during mitosis has long been a central question of cell biology, but one of the least accessible. In a report on page 206 of this issue¹, Hyman and Mitchison now demonstrate the force-producing capacity of the kinetochore, the portion of the chromosome required for movement. With this advance has come a surprise: the direction of force production is reversible.

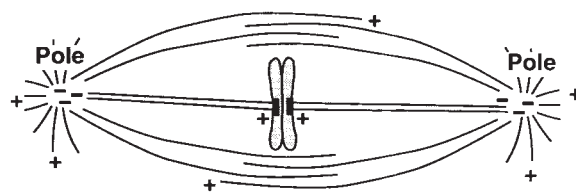
Microtubules provide the framework for the mitotic spindle, the structure responsible for chromosome separation. The microtubules are polar polymers, oriented with their so-called minus ends at the spindle poles and their plus ends overlapping in the centre of the spindle (see figure), and chromosomes attach to them by their kinetochores. The motions of chromosomes within the mitotic spindle are complex (see my News and Views article of a year ago²). In those few cell types in which early mitotic movements can be clearly observed, the chromosomes gather towards the spindle poles, during the stage referred to as prometaphase, through a series of rapid, intermittent movements³. Subsequently, they shuffle towards the mid-zone of the spindle in a process known as congression, where they align to form the 'metaphase plate'. In a highly coordinated fashion, anaphase then begins with the splitting of the chromatid pairs and the slow, uniform migration of the chromosomes towards the spindle poles.

It has been suspected that the mechanochemical (or motor) proteins kinesin⁴ and cytoplasmic dynein⁵ have a role in chromosome movement, as these proteins provide force along microtubules in opposite directions and could account for movements of the chromosomes both away from and towards the spindle poles. Recently, cytoplasmic dynein was found by immunofluorescence microscopy^{6,7} to be associated with the kinetochore, further implicating this protein in particular in mitosis. But other evidence indicated that the assembly and disassembly of microtubules alone is sufficient to drive chromosome movement, implying that force-producing molecules might not be needed.

One approach to dissecting the forces involved in mitosis has been to combine purified chromosomes and microtubules *in vitro* and examine their behaviour. It is however technically difficult to observe chromosomes moving along microtubules, as the chromosomes are large and the microtubules relatively slight. What Hyman and Mitchison have observed is the movement of microtubules against chromosomes. To detect this

movement and to define its direction, the microtubules were assembled from a mixture of fluorescently labelled and unlabelled tubulin subunits in such a way that the minus and plus ends could be distinguished by the intensity of their fluorescence.

Many of the microtubules became associated with the kinetochore. Dramatically, in the presence of ATP, they travelled across



The mitotic spindle showing orientation of the microtubules. Plus ends (+) of microtubules impinge on kinetochores, which are shown before the onset of chromosome splitting and anaphase separation.

the face of the kinetochore, movement being towards the minus end of the microtubule and thereby corresponding to the poleward direction of chromosome movement in the mitotic spindle. The rate (28 $\mu\text{m min}^{-1}$) was much greater than that seen during anaphase, but corresponds to the rapid rates of migration of chromosomes towards the spindle poles during early prometaphase³. This may be understandable if, as the authors believe, the chromosomes are arrested in their prometaphase state (they are still paired, for example). The observed movements can be accounted for by cytoplasmic dynein, which has already been implicated in early prometaphase movement^{3,6,7}.

But now for the surprise. If the chromosomes and microtubules were combined in the presence of the ATP analogue ATP- γ -S, the characteristics of the microtubule movement changed: it was much slower (2.9 $\mu\text{m min}^{-1}$) and in the opposite direction. How can this be explained?

Protein kinases can use ATP- γ -S as a substrate. The thiophosphate group is not readily removed from proteins by phosphatases, and will, therefore, modify proteins almost irreversibly. Using a specific anti-thiophosphate antibody, Hyman and Mitchison found that incorporation of thiophosphate was localized to the kinetochore, which strongly suggests that phosphorylation of a component protein of the kinetochore is responsible for the reversal of direction. Because phosphorylation was observed with purified chromosomes and microtubules, the kinase may also be associated with the kinetochore, as may the phosphatase. The inhibitor 6-dimethylaminopurine interfered with switching, implicating the cell-cycle-regulated cdc2 kinase in the reaction, although additional work will be needed to demonstrate the

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involvement of this particular enzyme.

The new observations raise many questions. One suggestion by the authors is that the plus-end-directed force-producing activity might account for the congression of chromosomes towards the metaphase plate. As yet, however, there is no direct physiological evidence for a force-producing molecule in congression, though a search for such a molecule may now be in order. Nor is it likely that a plus-end directed motor protein could fully explain congression: at the same time that one kinetochore is moving away from the spindle pole, its partner is always moving in the poleward direction.

What is the nature of the plus-end-directed force-producing enzyme? Although the direction of movement is that expected for kinesin, the rate and substrate specificity of the activity are inconsistent with the properties of any known molecule¹.

Are the isolated chromosomes, which are prepared from cells blocked in mitosis by microtubule-disrupting drugs, indeed arrested at a unique stage of the cell cycle? This is difficult to answer with certainty, and the properties of the kinetochores could conceivably reflect a combination of mitotic stages.

How can the involvement of a cell-cycle-regulated protein kinase be reconciled with the observed reversals in the direction of chromosome movement? A role for such a trigger in initiating anaphase seems likely, but there is at present little evidence for such a switch regulating the direction of force-production during the earlier stages in mitosis.

Finally, and perhaps of greatest interest, there is the matter of whether or not the force-producing activities detected in the isolated chromosomes are also involved in anaphase movement, or whether there is a distinct anaphase motor yet to be detected. Cytoplasmic dynein is clearly a front-running candidate for this job, and it is tempting to speculate that the function of kinetochore phosphorylation is to restrain the force-producing activity of this molecule until the onset of anaphase.

The ability to manipulate the components of the mitotic apparatus may now have moved ahead of our understanding of the detailed movements of chromosomes in the cell. But a major expansion of our understanding of mitosis seems imminent. □

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Consider the coiled coil . . .

Henry R. Bourne

How does a mutant tumour suppressor gene lead to colon cancer? Kinzler *et al.*¹ have identified a candidate for such a gene and propose that its normal protein product somehow interacts with signalling pathways mediated by G proteins. Further sequence gazing indicates that the protein belongs to the growing family of filamentous proteins that form coiled coils. Both observations point to obvious experiments.

The gene, dubbed *MCC* (mutated in colorectal cancer)¹, is the most recent in a series of discoveries that has made colon tumours a leading genetic model for understanding the initiation and progression of human cancer. Colon tumours progress from hyperproliferative lesions to form adenomas (colonic polyps) and, eventually, malignant carcinomas. As is probably the case for most kinds of cancer, the stepwise progression to malignancy results from serial accumulation of oncogenic mutations. The difference with colon tumours is that Bert Vogelstein and his colleagues have already identified many of the relevant mutant genes, including a *ras* proto-oncogene and two tumour suppressor genes, one that encodes a putative cell-adhesion protein and another that encodes a nuclear protein, p53 (see ref. 2 for review).

Now Vogelstein's team, working with that of Y. Nakamura, R. White and others, suggests that *MCC* is a third tumour suppressor gene¹. The search for *MCC* exploited two sets of genetic observations. First, the genetic defect in familial adenomatous polyposis (FAP), a dominantly inherited condition that predisposes patients to develop large numbers of colonic polyps — and, eventually, colon cancers — had been mapped to region 21 of the long arm of chromosome 5 (5q21). Second, a substantial proportion of sporadic colon tumours (in patients without FAP) have lost DNA sequences in and around 5q21. Kinzler *et al.* used these genetic deletions in sporadic tumours as guideposts to identify *MCC*.

The gene that has undergone mutation in FAP patients may turn out to be *MCC*, although this has not yet been documented and the authors are appropriately cautious about this point. If so, discovery of the normal function of the *MCC* protein will solve an apparent genetic paradox. On the one hand, the presence of 5q21 deletions in sporadic tumours implies that loss of function of a gene in this region contributed to formation of these neoplasms; by hypothesis, this is the *MCC* gene. On the other hand, the hundreds or thousands of colonic polyps in FAP patients have not lost 5q21 DNA; instead, the predisposition to develop these polyps results from mutation of a single allele of the FAP/*MCC* gene. Thus, mutations in *MCC* must act in a dominant fashion in individual cells of FAP patients.

How could a mutation in one copy of the gene responsible for FAP cause polyposis? Kinzler *et al.* discovered a striking sequence similarity between 19-residue stretches of amino-acid sequence in *MCC* and the m3 muscarinic acetylcholine receptor (mAChR), which mediates acetylcholine stimulation of phosphoinositide-specific phospholipase C via a G protein. Nine of the 19 residues, including six consecutive amino acids, are identical in the two proteins. In the m3 mAChR, these conserved amino acids specify the particular G protein — probably G_q (ref. 3) — with which the receptor interacts, and therefore the signalling pathway it initiates. Indeed, transfer of nine residues of the m3 mAChR (including four of the six consecutive amino acids that are identical in *MCC*) into the m2 mAChR makes the latter receptor able to stimulate production of phosphoinositide-specific phospholipase C (ref. 4).

Kinzler *et al.* suggest that the corresponding amino-acid sequence in *MCC* also contributes to a G protein binding site, but do not propose an explicit hypothesis relating *MCC* mutations to progression of sporadic colon tumours or to the FAP phenotype. One simple notion (Fig. 1) is that the *MCC* protein normally binds to and inhibits G_q (and other G proteins activated by receptors like the m3 mAChR). In this way, *MCC* would inhibit signalling through the Ca²⁺/phosphoinositide pathway, which is mitogenic in many cell types. Thus genetic inactivation of *MCC* in FAP or in sporadic colon tumours would 'derepress' signalling through G_q and phosphoinositide-specific phospholipase C, thereby enhancing mitogenesis.

This hypothesis can be tested: over-expression of *MCC* protein in cultured cells should inhibit stimulation of phosphoinositide-specific phospholipase C by agents that act through receptors linked to G_q, and pure G_q should bind to the *MCC* protein *in vitro*.

A precedent for the hypothesis depicted in Fig. 1 has been found in *Saccharomyces cerevisiae*, where the CDC39 protein has been suggested to attenuate activity of the G protein that mediates the response to mating pheromones⁵. Are the *MCC* and CDC39 proteins similar? I asked Aaron Neiman, the student who carried out these studies on CDC39, to compare the 829-residue amino-acid sequence of *MCC* to that of CDC39 (he had obtained the sequence from Steve Reed). Disappointingly, the sequences did not at all resemble one another.

Fortunately, Neiman did not stop there. He asked his computer to list proteins in the database with primary structures similar to that of *MCC*. "I'll bet *MCC* is a coiled coil protein", he said, pointing to the proteins at the top of the list: myosins, keratins, nuclear

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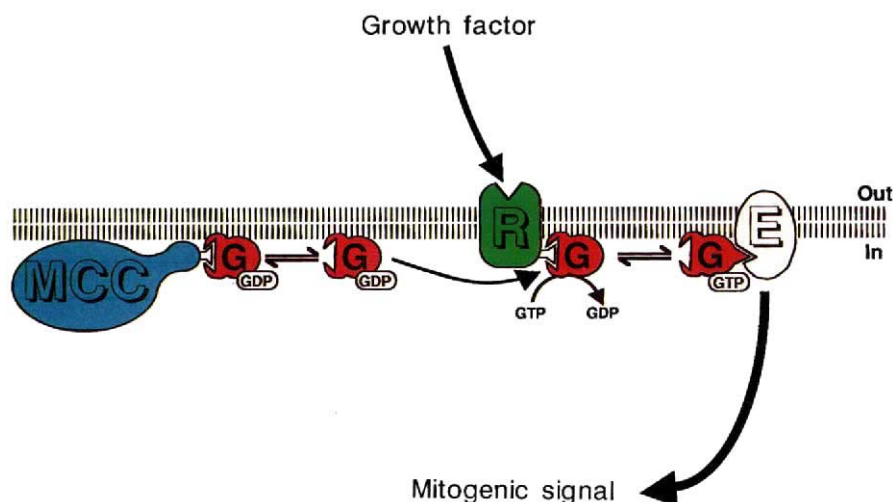


FIG. 1 Hypothetical role of MCC (mutated in colorectal cancer) protein as a G-protein inhibitor. The MCC binds the G protein (G), sequestering it from receptor (R), thereby preventing activation of the effector (E).

lamins, the *Drosophila* BicD protein, vimentin and other intermediate-filament proteins. Although functionally diverse, these proteins share the ability to form rod-like homodimeric complexes (and often higher-order oligomers) in which long regions of α helix wrap around each other in a coiled coil configuration^{6,7}. The amino-acid sequences of proteins in this family reveal long stretches of 'heptad repeats', in which the side chains of the first and fourth residues are hydrophobic; lacking prolines, these stretches of sequence can form uninterrupted α helices. The repeated hydrophobic side chains form a hydrophobic streak on one side of the helix; by allowing hydrophobic interaction between similar streaks on parallel α helices of two polypeptides, these repeats promote association of the two helices into a coiled coil^{6,7}.

This tell-tale structural motif appears in MCC as well (Fig. 2): the protein contains at least five proline-free stretches of heptad repeats, 42–133 amino acids in length. The two largest stretches are similar in size to the coiled coil regions of nuclear lamins⁶. As in the lamins, these putative α helices connect relatively hydrophilic regions, of varying size, whose proline residues and lack of heptad repeats make them unlikely to form coiled coils.

The distribution of heptad repeats suggests a kink-and-rod structure (Fig. 2). Each end of an MCC molecule would be a globular protein domain; these would be connected by a series of rods (the α helices) interrupted by small kinks (the short proline-rich regions between the helices). The rod regions of known coiled coil proteins extend approximately 15 nm per 100 amino-acid residues⁷. By this yardstick, the putative coiled coils in an imaginary extended MCC homodimer (Fig. 2c) could add up to 54 nm.

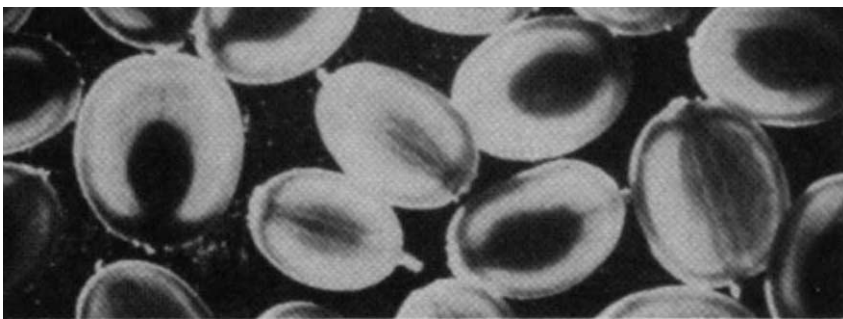
What does the structure proposed in Fig. 2 tell us about the function of the MCC protein? Coiled coils seem to serve as relatively stiff structural 'spacers' that specify distances between other proteins, which bind to the

globular domains at either end of the protein, and perhaps to some of the kinks in between. By associating into higher-order oligomers, they may form macromolecular structures that organize the cytoplasm (intermediate

filaments) or the nuclear membrane (lamins). They may be stable structures (muscle myosin) or fall apart and come back together in a precisely regulated fashion (as with the nuclear lamins before and after mitosis). The diversity of other members of the coiled coil protein family does not point to a specific function or cellular location for MCC. Nonetheless, cell proliferation is often regulated by cell shape and physical constraints imposed by other cells, making it easy to imagine that genetic disruption of a filamentous structure might somehow derepress a mitogenic signal.

The proposed coiled coil structure for MCC may help to explain the relation between 5q21 deletions in sporadic colon cancer and inheritance of polyposis — without evident deletions — in FAP. Because the MCC protein functions as a homodimer (or even a higher order multimer), recessive and dominant mutations in MCC can create functional deficits of widely differing severity. Point mutations that impair association between normal and mutant MCC polypeptides might cause mild deficits; large deletions of a single MCC allele could

Seeing through the fruits of deception



THE practice of deception is by no means confined to sentient organisms, but it rarely reaches the sophistication claimed by A. R. Zangerl and colleagues for the wild parsnip (*Evolutionary Ecology* 5, 136–145; 1991). This plant (*Pastinaca sativa*) produces fruits that lack viable seeds within the inflorescence (termed parthenocarpy) and the authors propose that the advantage to the plant of this apparent profligacy is that predators eat them rather than the viable fruits.

The regular occurrence of parthenocarpy suggests that the practice has some selective value for the plants that indulge in it. One proposal is that the rejection by the parent plant of certain of the fertilized ovules in the inflorescence provides it with a degree of mate choice, permitting the selective survival of favoured seeds. But observations on the fruits of the wild parsnip have led to an alternative idea, based upon the behaviour of larvae of the parsnip webworm (*Depressaria pastinacella*). This specialized lepidopteran fruit predator is shown by Zangerl *et al.* to prefer the parthenocarpic fruits to the viable ones, even though the former contain no embryo and no endosperm, and their consumption results in larval growth rates only one third of those that are possible when viable fruits are eaten.

Evidently there is some characteristic of the parthenocarpic fruit that makes it more attractive to the webworms. Visually it is difficult to distinguish between the two (except when lit from behind artificially, when the parthenocarpic fruits are seen to be empty — see picture), but the webworm selects its food by tasting the surface of the fruit and is particularly sensitive to deterrent furanocoumarins. These are about twice as abundant in the viable fruits, thus making the parthenocarpic ones more palatable.

The production of defunct fruits by the parsnip is energetically expensive and is unlikely to be the outcome of herbaceous altruism. Some plants produce non-functional fruits that serve to sustain insects (as in the case of figs and fig wasps) but only when the insect has some clear value to the plant (such as pollination). In this case, there is no evident advantage to the plant in maintaining webworm populations. Zangerl *et al.* conclude that the empty fruits serve as decoys, and perhaps the most remarkable feature of their observations is that the plant gains selective benefit from the allocation of its energy reserves in this way. The evolutionary path of deception is clearly a profitable one and some plants have not been slow to follow it.

Peter D. Moore

Dead right

M. B. Coleman *et al.* (*J. biol. Chem.* **266**, 5798–5800; 1991) show how a misdiagnosis can be posthumously exposed: doctors, it seems, can no longer safely bury their mistakes. The Indianapolis variant of haemoglobin gives rise to a highly haemolytic β -thalassaemia. The mutation was reported as $\text{cys}^{6112} \rightarrow \text{arg}$. But later this same substitution was found to attach to a milder condition, implying that the Indianapolis mutation had been incorrectly assigned. The two patients were by then dead, but a bone-marrow slide yielded DNA enough for detection of the mutation: leucine at $\beta 106$ had become arginine. Coleman and colleagues have renamed the (perhaps now extinct) variant haemoglobin Terre Haute.

Size Is Important

MANY kinds of animals readily change sex, and according to one hypothesis it is likely to happen if fecundity increases with age or size in one sex more rapidly than in the other. Species of polychaete worm are good subjects for testing this idea (A. Berglund, *Evolutionary Ecology* **5**, 128–135; 1991). *Ophryotrocha puerilis* males respond to the mid-life crisis by switching sex, but sex-change in the almost identical *O. labronica* is rare. Female choice may in fact be responsible: *O. labronica* females prefer large males, *O. puerilis* female small ones, presumably rejecting the larger males because they are in imminent danger of changing sex and competing with them — as females — for mates. On reaching a certain size, then, males respond by making the ultimate sacrifice.

Ring ratings

THE buzz word associated with benzene is aromaticity — the ability to shuffle charge through its bonds for added stability. But what of its inorganic analogues? ask W. H. Fink and J. C. Richards (*J. Am. chem. Soc.* **113**, 3393–3398; 1991). Borazine, a ring of alternating boron and nitrogen ($\text{B}_3\text{N}_3\text{H}_6$), is well known, but is chemically very different from benzene, a difference that is put down to a lack of aromaticity. P. Power *et al.*, colleagues of Fink and Richards, recently synthesized boron–phosphorus analogues, but bulky substituents for the hydrogen atoms confuse the picture of charge distribution around the ring. Fink and Richards resort to the computer and quantum calculations for a simpler answer, and suggest that boron–nitrogen and boron–phosphorus analogues are half as aromatic as benzene, and that aluminium–nitrogen analogues hardly rate at all.

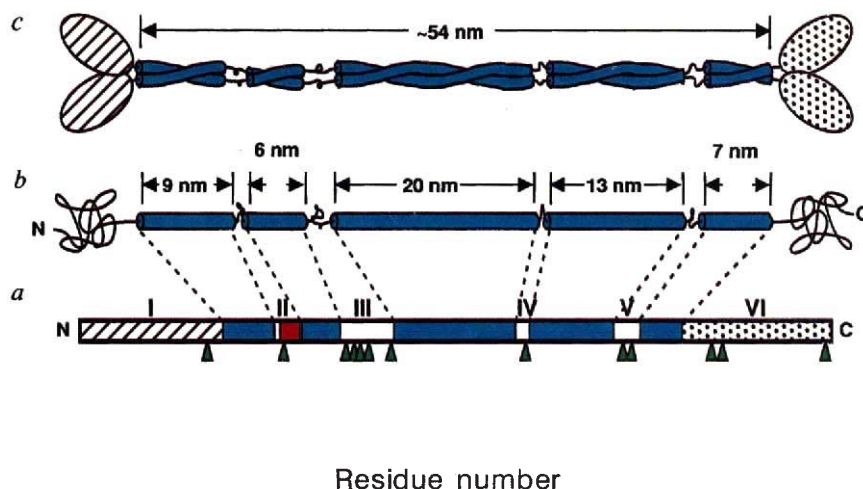


FIG. 2 Rod-and-kink model of MCC structure. a, Location of sequence motifs in primary structure — blue, heptad repeat ('coiled coil'); red, sequence that is similar to that in the m3 muscarinic acetylcholine receptor; green, prolines. b, Predicted domain structure of MCC monomer. c, Coiled coil structure of homodimer.

produce a 50 per cent decrease in MCC concentration and an intermediate functional deficit; strong dominant negative mutations could come close to abolishing MCC function altogether. This may explain how FAP causes colonic polyposis of different severities in different families, and in some families produces additional heritable defects, including abnormal development of teeth and bone and increased incidence of cancers in skin, brain and other organs, as well as cancer of the colon⁸.

Mild MCC alleles might also account for the increase in incidence of colonic polyps found in first-degree relatives of patients with 'sporadic' colon cancer, consistent with inheritance of a dominant mutation with variable penetrance⁹. Inheritance of such mild defects in one MCC gene might set the stage for tumour progression caused by subsequent deletion of the normal gene, a somatic mutation which would further diminish MCC function. Such a process could explain the substantial (20–30 per cent) incidence of 5q21 deletions in apparently sporadic colon cancers. Similar reasoning could account for the detection¹⁰ of 5q21 deletions in colonic cancers (but not adenomas) of FAP patients as well.

Man-made dominant negative mutations might help to identify what MCC does in normal cells. Expression of a dominant-negative¹¹ mutant MCC protein (for example one lacking either the N- or C-terminal globular domains postulated in Fig. 2) would presumably impair MCC's spacing function. The resulting phenotype could reveal what that function is. Precedents include man-made mutations in nuclear lamins¹², dominant paralyzing mutations in a myosin heavy chain gene of *Caenorhabditis elegans*¹³, and myosin mutations associated with familial cardiomyopathy in humans^{14,15}.

From the genetic point of view, MCC in this model acts like another tumour suppressor gene, *p53* (ref. 16). Mutations of MCC in familial adenomatous polyposis,

like *p53* mutations inherited in cancer-prone families with the Li-Fraumeni syndrome^{17,18}, induce tumours after a delay of several decades. Alternatively, somatic mutations in either the MCC gene or the *p53* gene can contribute to tumour progression. As Vogelstein noted in his News and Views article¹⁶, "it is not the time of occurrence of mutations, but rather their accumulation, that is most important in tumour development".

Now is the time to lay your bets. Is MCC a G-protein inhibitor or a coiled coil? Weighing long stretches of heptad repeats against six identical amino acids, I am betting on the coiled coil. You may hedge your bets, of course, because the two hypotheses are not mutually exclusive. The MCC sequence that resembles the m3 muscarinic acetylcholine receptor is located in a kink between postulated rod-like regions (Fig. 2), where it could easily serve to attach G_i to a macromolecular structure somewhere near the cytoplasmic face of the plasma membrane. □

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Slow-motion galactic birth

Joseph Silk

THE timescale over which primordial material collapsed to form our Galaxy is of considerable cosmological significance. The debate is hinged on whether the collapse occurred over a 'dynamical' timescale, which would require all the old stars in the Galaxy to have formed within an initial free-fall time of less than a billion (10^9) years, or whether the early collapse was a more gradual process. Perhaps our Galaxy contracted over several billion years: if this were the case, there are noteworthy, even revolutionary, implications for our picture of galaxy formation. New observations of two globular star clusters by R. J. Dickens *et al.*, reported on page 212 of this issue, strengthen the view that there is a spread of about 3 billion years in the ages of some of the oldest stars in our Galaxy.

The significance of globular clusters, gravitationally bound associations of up to a few million stars, is that they are thought to be the oldest objects in our Galaxy. The two chosen for the new study, NGC288 and NGC362, have long been suspected to differ in age by 3 billion years. This inference is based on the colour-luminosity plot (Hertzsprung-Russell diagram) of the stars in the clusters: specifically, the point at which the stars depart from the main, hydrogen-burning sequence. In older clusters, the more massive, brighter stars will have exhausted their hydrogen cores and only the fainter, less massive stars will remain on the main sequence. The two clusters appear to have the same metallicity, a parameter which affects the turn-off luminosity at a given age and so might thereby introduce further uncertainty into any interpretation of difference in turn-off. This pair of clusters is therefore an ideal candidate for an age difference between two stellar systems that formed when the Galaxy first underwent collapse.

However, previous studies made unwarranted assumptions about the metallicity of these clusters. Astronomers loosely group C, N and O, as well as Fe, under the rubric of 'metals', and there is reason to believe that in old stars in the galactic halo, the O/Fe abundance may differ from the solar value. Indeed at $[\text{Fe}/\text{H}] < -1$ (a compact notation that means Fe/H is less than 10 per cent of the solar value), $[\text{O}/\text{Fe}]$ is about 0.5 (an enhancement of 3) relative to the solar value for field halo stars. A factor of 3 increase in O/Fe decreases the age by 2 Gyr from that derived for a given turn off assuming the solar O/Fe ratio. The previous studies of globular clusters have not directly measured C, N, O and Fe in the same stars, but assumed a solar ratio of C, N, O to Fe. Moreover, the globular-cluster stars measured are stellar giants whose surface abundances may be contaminated by the

nuclear reaction cycle involving C, N and O, which occurs in the stellar core. There is therefore serious cause for concern that the ages (and age differences if O/Fe varies from cluster to cluster) may be overestimated.

The new study obtains accurate C, N, O and Fe abundances for samples of stars in the two globular clusters. The CNO cycle reactions can modify the abundances of C, N and O, but the sum C+N+O is conserved. The spectroscopic study reveals that although $(\text{C+N+O})/\text{Fe}$ is constant from star to star and from cluster to cluster, O varies markedly with, for example, C. The variations with C and with N in NGC362 confirm the trend expected for material that has undergone CNO cycling in stellar cores and then been mixed with surface matter. But NGC288 shows no evidence of such surface contamination in its giant stars. The two clusters are inferred to have had very different histories of mixing. The unmixed stars are enhanced in $[\text{O}/\text{Fe}]$ by about 0.2, a slightly smaller enhancement than seen for halo-field dwarfs at the same metallicity.

The moral of this investigation to observers is: take heed — do not use O/Fe in a small sample of stars (an inevitable restriction because of the long integration times needed to acquire high-resolution spectra) to infer global trends of $[(\text{C+N+O})/\text{H}]$, this ratio being the key to age, or relative-age, determinations. Lack of

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knowledge of $[(\text{C+N+O})/\text{H}]$ made previous attempts at age determinations uncertain by 3 Gyr or more.

Dickens *et al.* find that the two clusters have almost identical abundances of $[(\text{C+N+O})/\text{H}]$, as well as of $[\text{Fe}/\text{H}]$ and conclude that the clusters therefore do differ in age by 3 ± 1 billion years. The age difference comes tantalizingly close to showing that the Galaxy took longer than the free-fall time, about 1 billion years, to collapse. The globular clusters, because of their eccentric orbits, must have formed during this collapse. Gaseous dissipation of the energy released during contraction provides a means of slowing the collapse. Most models of galaxy formation require the round spheroid of the Galaxy to collapse dynamically on a free-fall time. A similar scheme applies to the formation of elliptical galaxies. The longer timescale, if confirmed, should inspire a new approach to galaxy formation theory.

Astronomers are left with another puzzle; the two globular clusters have very different horizontal branch (post-main sequence) morphologies: NGC362 has mostly red giants, whereas NGC288 has predominantly blue giants. A second parameter, other than age, is needed to explain this, and the new study shows that the $[(\text{C+N+O})/\text{Fe}]$ abundance is not the culprit. Theorists and observers alike have much work left to do before the formation of globular clusters is fully understood. □

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Iceland melting dynamics

Rob Ellam

RECENT years have seen a transformation in the way petrologists regard melting processes that occur in the Earth's mantle to generate the magmas that crystallize as basalts at the surface. The breakthrough has come largely from better understanding of the physics of melting, and above all, from the recognition that even rather small melt fractions are likely to form an interconnected network within the partially molten mantle, allowing rapid segregation of melt from the solid matrix¹. Thus, old ideas of static melt regimes with large melt fractions (10–20 per cent by volume) remaining in contact and in chemical equilibrium with the residual solid matrix seem redundant and have been replaced by dynamic melting models in which small melt fractions (less than 2 per cent) are continuously extracted from an upwelling column of mantle². On page 201 of this issue³, Elliot *et al.* present highly coherent major- and trace-element and isotope-ratio variations in Icelandic basalts that appear to indicate the operation of dynamic melting beneath that area.

Two of the most important parameters that control melting processes in the mantle are its temperature and the amount of extension of the lithosphere, the rigid plate above the convecting mantle (asthenosphere). Lithospheric extension allows upwelling of the underlying asthenosphere causing melting through adiabatic decompression. Clearly, for a given extension or stretching factor, the extent of melting will be greatest where the mantle is hottest. Iceland sits astride the North Atlantic ridge where new oceanic lithosphere is created and the stretching factor is therefore effectively infinite. However, beneath Iceland the upwelling mantle is some 200 °C hotter than that found beneath normal ridge segments. So adiabatic upwelling generates more melt and the most noticeable consequence is the great thickness of the Icelandic crust (not least, the existence of Iceland itself) compared with the average thickness of oceanic crust (typically 7 km; ref. 4).

Melts generated from the hot plume of mantle beneath Iceland are also geochemi-

cally distinct from normal mid-ocean-ridge basalts (MORB), being relatively enriched in the so-called incompatible trace elements, such as potassium and lanthanum, that partition most readily into the melt phase when the mantle undergoes partial fusion⁵. Similarly, the Iceland plume is well known for a characteristic isotope signature, having higher $^{87}\text{Sr}/^{86}\text{Sr}$ and lower $^{143}\text{Nd}/^{144}\text{Nd}$ ratios than the adjacent ridge sections^{6,7}. Now Elliot *et al.*³ present new data on highly magnesian basalts (picrites) from two areas of Iceland. A simple prediction is that these ought to approach the plume composition. This follows because MgO-rich mantle minerals are more refractory than their FeO-rich equivalents, so that early magmas are iron-rich, giving way to higher MgO melts at the relatively large melt fractions likely to be associated with melting in a hot plume. But the picrites show quite opposite characteristics, being highly depleted in incompatible trace elements and having Sr, Nd and Pb isotope ratios that approach those of the adjacent MORB^{3,8}. Paradoxically, it seems to be the basalts with lower MgO content that represent the isotope and trace-element contribution from the plume.

Correlations

The keys to understanding this problematic observation appear to be the highly coherent correlations between iron content and trace-element and isotope parameters in the Iceland basalts: elevated iron content is associated with incompatible trace-element enrichment and a low ratio $^{143}\text{Nd}/^{144}\text{Nd}$. This is a critical observation, because the iron content of magmas is thought to be an important index, being relatively insensitive to the degree of partial melting, but extremely sensitive to the depth of melt segregation. In short, the higher-pressure melts are iron-rich in comparison with their low-pressure counterparts.

In a dynamic melting column, the onset of melting of the upwelling is followed by continued melting during the remainder of the ascent, causing progressive depletion of melt-forming constituents, for example iron. The column is in steady state, the refractory residue leaving the top of the column being continuously replaced by new fertile mantle entering the base. With the relatively new constraint that melt fractions as small as 2 per cent are able to separate efficiently from the solid matrix, it is clear that these instantaneous melts need not equilibrate further with the upwelling mantle.

Rather, each instantaneous parcel of melt will preserve the iron content appropriate to its separation pressure. In addition, there will be a relationship between the FeO and the trace-element composition as the lion's share of the incompatible-element inventory of a particular portion of mantle is repeatedly scavenged by the early, deep, iron-rich melts. Thus, far from approaching the plume contribution, the iron-poor picrites are taken to

represent very shallow melts, perhaps the last gasp of melt from what is now very refractory mantle as it leaves the top of the melting regime.

Of course, in practice it is likely that the instantaneous melts will subsequently mix and the final magma will represent some average of melts from different portions of the dynamic column. But, by now the die has been cast and the characteristic relationships between iron and the degree of trace-element and isotope enrichment have been established.

Mixing

However, dynamic melting alone is not able to explain the observed range in isotope ratios. Some form of mixing is required, for example, between relatively low- $^{143}\text{Nd}/^{144}\text{Nd}$ plume material and (depleted) mid-ocean-ridge mantle with high $^{143}\text{Nd}/^{144}\text{Nd}$. Entrainment of depleted mantle by the plume is quite feasible, and will have the effect of mixing MORB into the plume-derived melts. It is difficult to see why this should be related to the depth of magma segregation, that is, why it should correlate with iron content — unless the effect of mixing MORB into the various melts of the dynamic column is considered. Clearly the MORB magma will exert more leverage on the Nd isotope composition of mixtures of MORB and highly (Nd) depleted shallow-level melts, than on mixtures of MORB and relatively Nd-rich melts from the bottom of the melting column. Therefore the relationship between FeO and $^{143}\text{Nd}/^{144}\text{Nd}$ need not represent an Nd isotope gradation in the source region, but instead simply an increasing response to MORB contamination as the trace-element signature of plume magmas decays with repeated episodes of melting.

Although Elliot *et al.* have chosen to concentrate on the well-exposed and well-documented magmatism of Iceland, where the upwelling mantle is both hot and trace element-rich, there can be little doubt that similar processes are likely beneath more normal segments of oceanic ridge. The challenge will be to recognize these effects elsewhere, and the lesson must be that to do so requires judicious combination of a variety of geochemical data coupled with constraints from geophysics. □

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Desert shield

THE world's deserts are inexorably spreading. Stripped of its plant cover by over exploitation, the soil is exposed to tropical sunlight. This breaks down its organic matter and reduces its capacity to hold water. New plants cannot get a foothold; the bare, Sun-heated soil tends to reduce cloud formation above it, and rapidly evaporates the rain and dew which do manage to fall. Desertification is geologically rapid. A few thousand years ago much of the Sahara desert was still productive, and the Amazon basin is going the same way with dizzying rapidity.

Daedalus now plans to reverse the process. He has been inspired by those spot lamps whose multilayer-dielectric reflectors reflect their visible light with high efficiency while transmitting their infrared. Modern, high-speed evaporative deposition could lay down such a reflective layer on thin polyester film on quite a large scale. Spread over the soil of the desert, the resulting sheet would reflect away most of the incident sunlight, while permitting the infrared of the heated soil to escape freely. The film and the ground beneath it will rapidly cool down. Rain and dew will be deposited much more readily.

As the welcome water gathers in puddles on the film, an array of small holes will drain it onto the ground. Water vapour, however, will diffuse upwards through the holes much more slowly. Relative humidity beneath the film will rise. To give vegetation a start, some sort of initial mulch and ground-cover must be provided. Impressed by the dense weed-growth on so much trash-strewn waste land, Daedalus proposes to spread the developed world's garbage over the desert.

The dumping of waste in the Third World, a covert activity now deplored by high-minded environmentalists, will thus become an open trade benefitting both parties. An area on the edge of a desert will be liberally strewn with old newspaper, crumpled packaging, smashed furniture, ashes and tin cans. It will be well fertilized with sewage sludge, sown with an appropriate mix of weed-seeds, covered with selective-reflector film, and left.

In the cool, humid, attenuated daylight beneath the film, a luxuriant plant ecology should rapidly sprout forth on the rich and varied environment of damp trash. Soon it will be sturdy enough to survive and thrive on undiluted daylight. The adjacent stretch of desert will then be seeded with junk, the reflective film will be transferred to it, and the process will continue. Slowly, like a wound healing in from the edges, the desert will shrink. Its primary ecology, weeds over junk, will not be much use; but it will slowly diversify and stabilize into potential farmland. The recovery of our battered planet will be under way. David Jones

Dispersal via whale bones

SIR — Smith *et al.*¹ reported the occurrence of chemosynthetic invertebrates, especially molluscs, associated with whale bones on the Pacific Ocean floor. More recently, Squires *et al.*² have given an account of fossil whale carcasses from Oligocene deep-water sediments also associated with chemosynthetic molluscs, and discussed the possibility of whale carcasses acting as stepping stones for the wide dispersal of chemosynthetic invertebrates over a prolonged period. But Squires *et al.* argue that carcasses large enough to act as benthic stepping stones have existed in the Pacific Basin only since the late Miocene, that is, for the past 11 million years. We believe the idea that only whale carcasses can act as refugia is unduly restrictive. The oceans have contained large marine tetrapods since at least the end of the Triassic, in other words for around 200 million years. By large, we mean animals with body lengths (conservatively estimated) of up to 10 m. In addition, large fishes also up to 10 m long are known from at least as early as the Middle and Upper Jurassic to the present day.

Associations of fossil marine-reptile skeletons with abundant invertebrates have been demonstrated. For example, a specimen of the ichthyosaur *Ophthalmosaurus* was associated with specimens of the small scaphopod *Prodentalium*³, a taxon reported associated with Eocene methane vents⁴; and a complete plesiosaur skeleton was associated with articulated valves of *Nicaniella* (*Trautscholdia*) sp. from the Oxford Clay (Callovian, Middle Jurassic), an organic rich mudrock of shallow water facies. This deposit is itself rich in early diagenetic pyrite, indicating considerable sulphate reduction in the top few millimetres of the sea floor.

If vertebrate carcasses are important in the dispersal of vent faunas, then such refugia may have occurred from at least the end of the Middle Triassic, when large ichthyopterians first appeared, or from the end of the Triassic, when large sauropterians made their first appearance. The ichthyosaurs disappeared during the Middle Cretaceous, but sauropterians and mosasaurs remained important parts of the marine fauna until the end of the Mesozoic. Gigantic fishes have been present in the seas since the Devonian. Large fishes, including teleosts and elasmobranchs, spanned the Cretaceous/Tertiary boundary⁵, and may have provided refugia before the arrival of cetaceans in the Eocene.

Nevertheless, caution should be exercised when examining fossils associated with vertebrates preserved in concretions. Early diagenesis around carcasses may preferentially preserve calcareous invertebrates that may be absent from the surrounding sediment due to diagenetic removal. In addition, preparators of vertebrate fossils may inadvertently destroy invertebrates. We suggest that palaeontologists look more closely at the

skeletons of large marine vertebrates of all sorts, and especially their associated invertebrates.

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Human circadian rhythms

SIR — Winfree in his News and Views¹ on our paper² outlined the development of the theory that underlies the concept of the phase-free singular condition of a physiological rhythm generator, but in doing so he understated his own contribution. He has articulated lucidly the mathematical necessity of the singular state³, developed an experimental protocol (the singularity trap) by which the timing and strength of the critical stimulus can be bounded, and applied this protocol to a circadian rhythm of *Drosophila melanogaster*⁴. But Winfree's use of the terms "subjective midnight" and "subjective noon" to calibrate the response of the human circadian clock to light could be misunderstood.

We have shown² that, for persons reasonably well entrained to the conventional day the critical light stimulus is centred at 4:00 to 5:00 a.m. This time also represents the transition between those light-induced phase shifts that retard the circadian clock (light episodes before 4:00 a.m.) and those that advance it (episodes after 5:00 a.m.). The complementary time for transition from advances to retardations is 4:00–5:00 p.m. Thus, Winfree's subjective midnight is, for humans, about 4:30 a.m., whereas subjective noon is about 4:30 p.m. That inconsistency may in part be because Winfree has chosen to use the *onset* of the stimulus as its reference time. For the relatively brief light stimuli which are effective in many insects and nocturnal rodents, the distinction between onset time and mid-stimulus time is small. But for the 5- to 9-hour range of stimuli that we used, consistency is obtained only if the centre of the stimulus episode is taken as its reference. This was also the practice of Gander and Lewis⁵ when using 4- to 16-hour light episodes as stimuli for *Rattus exulans*.

We urge that the 'subjective noon' and 'subjective midnight' nomenclature be avoided with reference to humans because of its susceptibility to misinterpretation by the untutored reader. Indeed, it can be misleading even to experts. Before any data were available on human resetting by light, Daan and Lewy⁶ conjectured that the human response might qualitatively resemble the well-documented type 1 response of nocturnal rodents. They consequently assumed that subjective midnight for humans was midnight, thereby misestimating the true critical time for humans by 4 to 5 hours.

Like Winfree, we are struck by the uniformity of response characteristics across species, but the precise relation of critical time to clock time depends both on the species and its environmental light exposure. In humans this critical time is closer to dawn than to midnight.

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The right way

SIR — In our recent paper on information storage using films of an azobenzene derivative¹, we proposed a mechanism to explain the experimental results which differed from that published by us in an earlier, preliminary paper². After detailed investigations, we have concluded that the earlier mechanism was not adequate. It is the new mechanism proposed in ref. 1, and described in more detail in ref. 3, that provides the possibility of photo-electrochemical information storage.

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Mitochondrial base prediction

SIR — Goto *et al.* reported¹ that a heteroplasmic mutation in the mitochondrial transfer RNA^{leu(UR)} gene is found in familial cases of the MELAS subgroup of mitochondrial encephalomyopathies. This mutation replaces a conserved A residue of the DHU loop of the tRNA with a G, and the authors suggest that this alteration to the structure of the rRNA may play a role in pathogenesis. Although this idea is perfectly plausible, the affected nucleotide pair has an additional, identified function, suggesting an alternative pathogenic mechanism.

The tRNA^{leu(UR)} gene of vertebrate mitochondrial DNA overlaps a transcriptional attenuator, functionally identified *in vitro* in human cells^{2,3} and highly conserved at the sequence level (11/13 nucleotides) in mouse, rat, cow, frog⁴ and chicken. This sequence functions bidirectionally⁵, and its action seems to be mediated by specific protein-binding^{2,3}. This is believed to be the principal mechanism regulating the relative synthetic rates of mitochondrial, ribosomal, messenger and transfer RNA in vertebrate cells, because the rRNA genes, plus those encoding tRNA^{phe} and tRNA^{val}, are found, in the transcription unit of the heavy strand, upstream of the attenuator. This is supported by observations that these two rRNAs are synthesized at much high levels than the remainder².

We suggest that a mutation in the attenuator could cause disease by an altered affinity for the protein factor(s) involved in termination, thus leading either to an excess or a deficiency in the rate of synthesis of different classes of RNA. This hypothesis has a clear and easily testable prediction, namely that the protein-binding characteristics of the attenuator, as well as the efficiency of transcriptional termination *in vitro*, should be altered by this base change.

It might also be expected that some mutations in the nuclear gene encoding the termination factor could have a similar effect on its binding affinity or specificity, resulting in symptoms indistinguishable from MELAS. Alternatively, other alleles of this gene could regulate penetrance by acting as suppressors of the mitochondrial mutation.

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Clues to action of cdc25 protein

SIR — The cdc25 protein is required to promote dephosphorylation of a specific tyrosine residue Tyr15 in p34^{cdc2}, which leads to activation of the p34^{cdc2}/cyclin B kinase and consequently to the onset of mitosis^{1–3}. Comparisons between the amino-acid sequences of cdc25 and of previously published protein tyrosine phosphatases (PTPases) have not revealed extensive

cdc25	1–	430	D	K	(17)	T	(10)	V	–	A	F	L	S	K	(6)	A	L	V	F	H	C	E	H	S	A	H	R	A	P	(3)	L	(15)	F	L	Y	–	Y	(5)	L	–	G
VH1	1–	60	D	K	(17)	T	(10)	V	–	A	F	L	S	K	(6)	P	V	L	V	H	C	A	A	G	V	N	R	S	G	(3)	L	(15)	F	L	Y	–	Y	(5)	L	–	G

Sequence comparison between *Schizosaccharomyces pombe* cdc25 protein and vaccinia virus VH1 protein phosphatase. Identical matches are boxed. Amino acids represented in bold in the cdc25 sequence are conserved among cdc25 homologues, whereas those in the VH1 sequence are conserved in protein tyrosine phosphatases. The line marks the 'signature sequence' containing the putative catalytic site.

similarities, and so it has been unclear whether cdc25 acts directly to dephosphorylate p34^{cdc2}. But we have found that the cdc25 protein shares a degree of similarity with a newly identified phosphatase which can dephosphorylate both serine and tyrosine residues⁴. This is the vaccinia virus protein VH1, which gives the highest score in the PIR protein database, after a search for similarities in sequence to the conserved amino acids among cdc25 homologues from yeast to human. A region of 92 amino acids in the C terminus of cdc25 shows 21 per cent identity to VH1 (see figure). This region includes a motif, HC (5) R, which is found in all PTPases and is thought to include the putative catalytic site. These cysteine (C) and arginine (R) residues are absolutely essential for phosphatase activity^{4,5}.

The possibility that the cdc25 protein might act directly to dephosphorylate Tyr15 in p34^{cdc2} gains further support from recent genetic and biochemical work investigating the role of cdc25. Fission yeast cells that are unable to divide because they contain a mutation in or are deleted for *cdc25+* can be rescued by overexpressing a T-cell tyrosine phosphatase¹. Also, purified, bacterially produced *string* protein (a *Drosophila* cdc25 homologue) can dephosphorylate partially purified *Xenopus* p34^{cdc2}/cyclin (ref 2); similar results are obtained using bacterially produced human CDC25 and highly purified p34^{cdc2}/cyclin obtained from starfish oocytes (ref. 3 on page 242 of this issue). In all cases *in vitro* dephosphorylation of p34^{cdc2} results in an increase in kinase activity. The similarities in sequence between cdc25 and the VH1 tyrosine/serine phosphatase obviously suggest that the cdc25 protein acts directly as a phosphatase. But until further enzymatic studies are performed it cannot be ruled out that cdc25 functions in another manner. For example, it could act noncatalytically, leading to irreversible phosphate transfer to the cysteine residue (thought to be the nucleophilic phos-

phate acceptor in the enzyme mechanism)^{3,4}. Such a stoichiometric mode of action might explain the dosage effects of cdc25 over onset of mitosis.

In vertebrate cells, dephosphorylation of both Thr14 and Tyr15 in p34^{cdc2} is required for the activation of the kinase (C. J. Norbury, J. J. Blow and P. N., manuscript submitted). It is therefore of interest that the cdc25 protein is similar in sequence to a protein phosphatase with activity against both tyrosine and serine (and thus probably threonine) phosphate residues. If the cdc25

protein has activity against both tyrosine and threonine phosphate residues, then it could dephosphorylate both Thr14 and Tyr15 bringing about onset of mitosis in vertebrate cells.

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Conscious or unconscious?

SIR — Goodale *et al.*¹ may have confused an important distinction relating to conscious and unconscious functions. They state that their evidence suggests that "... the visual processing underlying conscious perceptual judgements must operate separately from that underlying the 'automatic' visuomotor guidance of skilled actions ...". This implies a separation between processing pathways for conscious and unconscious functions generally.

Perhaps Goodale *et al.* based such a separation on the assumption that all the perceptual tests in which their subject failed represented tests of conscious awareness of the signal. But we have recently gained evidence for the possibility that such tests might be performed successfully by a normal subject even without awareness². Given electrical stimuli in the somatosensory (ventrobasal) thalamus, human subjects correctly detected the presence of a stimulus (in forced choice responses), even when they were completely

unaware of any sensation and were 'guessing'. The stimuli were all at the same pulse frequency and intensity; only the train durations were varied. Brief trains (about 250 ms) elicited correct detection with no awareness, whereas substantially longer trains (about 500 ms) were required to elicit correct detection with awareness. In this case the same afferent input pathway to sensory cortex subserved both the conscious and the covert (unconscious) detection of a signal.

The distinction in the findings by Goodale *et al.* may, therefore, be between perceptual information of a signal and visuomotor guidance of (skilled) movements towards a signal, rather than between conscious judgements and automatic guidance of a motion. Goodale *et al.* do conclude that their "observations indicate separate processing systems not for different subsets of visual information, but for different uses to which vision can be put". I agree, but wish to emphasize

that such a separation should not necessarily imply a parallel general separation between conscious and unconscious functions. This is an issue with potentially far-reaching consequences for the cerebral basis of conscious, subjective experience. It may be that one controlling factor for the transition between the conscious and unconscious features of information is simply the duration of appropriate neural activity, and that both features could (although not necessarily) then be a function of the same cerebral areas³.

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FK506 and protein kinase C

SIR — We and others have cloned binding proteins for the novel immunosuppressant FK506^{1–3}. These FK506-binding proteins (FKBPs) are active as peptidyl-prolyl *cis*–*trans* isomerases (PPIases), and accelerate the slow-folding phase of certain proteins *in vitro*; FK506 inhibits this enzymatic activity. Strikingly, another immunosuppressant, cyclosporin A, binds a different

identity to the *Neurospora crassa* FKBP. (The differences most probably result from the fact that the proteins were isolated from different organisms.)

We therefore conclude that PKC I-2 and FKBP are identical proteins. Protein kinase C is a Ca²⁺- and phospholipid-dependent key enzyme involved in signal transduction in a number of cells⁶. Two endogenous in-

```
Nc-FKBP  TIPQLDGLQIEVQOEGQGTRETRRGDNVDVHYKGVLTSGKKFDASYDRGEPLNFTVQGQO
PKC I-2   GVQVETISPGDGRTEPKRGQTCVHYTGMLEDGKKFSSSRDRNKPFFKVLGKQE
h-FKBP    -----M-----
```

```
Nc-FKBP  VIKGWDGLLGMKIGEKRLTIAPHLAYGNRAVGGIIPANSTLIFETELVGIGKVQKGE
PKC I-2  VIRGWEQGAQMSVQRAKLTIISPDYAYGATGHPGIIPPATLIFDVELL-----KLE
h-FKBP    -----H---V-----
```

Comparison of the amino-acid sequences (single-letter notation) of FK506-binding protein from *N. crassa* (Nc-FKBP; ref. 1), with bovine PKC-inhibitor 2 (PKC I-2; ref. 5) and with human FK506-binding protein (h-FKBP; refs. 2,3). Amino acids identical to both Nc-FKBP and PKC I-2 are shown in bold. In lane 3 (h-FKBP) only amino acids that are different between PKC I-2 and h-FKBP are shown.

PPIase, cyclophilin, and inhibits its enzymatic activity.

At first we noticed sequence similarity of FKBP to only one other protein, a cell-surface protein from the bacterium *Legionella pneumophila*, which causes Legionnaires' disease^{1,4}. But by screening data banks we have now found similarity of FKBP to an inhibitor of protein kinase C from bovine brain⁵ (PKC I-2; see figure). Bovine PKC I-2 shows 97 per cent amino-acid identity to the human and 49 per cent

inhibitors of bovine protein kinase C have been isolated and sequenced⁵. As FK506 seems to act as an immunosuppressant in lymphocytes by interfering with the Ca²⁺ signalling pathway, we speculate that FK506 may act through FKBP (PKC I-2) by interfering with the activity of protein kinase C.

On the basis of studies with *N. crassa* and *Saccharomyces cerevisiae* (ref. 7 and unpublished results), we conclude that it is not the inhibition of the PPIase activity but a complex between immunosuppressant/PPIase and a third component that is lethal for those cells. We now propose that this third component may be protein kinase C.

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Lake Nyos dam threat

SIR — About 55×10^6 m³ of water is impounded by a natural dam at Lake Nyos maar, Cameroon. This 40-m high dam, currently about 45-m wide at the top, is composed of pyroclastic ash deposited during the explosive formation of the maar. These pyroclastic materials are fairly well indurated at the surface but include deeper friable layers that have little resistance to erosion. Thus, headward erosion has reduced the width of the dam by several hundred metres since its formation as recently as 400 years ago¹. The dam's eventual failure could generate a flood that would imperil the lives of thousands of people downstream in Cameroon and Nigeria².

A field inspection of the dam in early December 1990 showed that erosion of the downstream face has continued since our last visit in 1987; a large block had fallen from the overhanging downstream face, possibly during the 1990 rainy season. Perhaps more ominously, the 'caves' on the downstream face of the dam are clearly the result of incipient seepage erosion (piping³) by lake water seeping through the dam. These caves extend at least 5 m headward into the dam, and the seepage erosion that has caused them will be the most likely cause of the eventual failure of the dam. The caves cannot be safely explored because they serve as outlets for CO₂-rich metalimnion lake waters seeping through the dam; thus, dangerous accumulations of gas may be present.

Underwater inspection of the lakeward face of the dam showed that it is also being eroded. The upper, more erosion-resistant surface of the dam has been undercut more than 2 m along the entire upstream face. Furthermore, the earlier-described 'joints'² on the upper surface are postulated as stress fractures related to dam geometry. Large blocks bounded by these fractures are ready to fall from the downstream face as the underlying poorly indurated ash deposits are eroded.

The seriousness of the situation is recognized by the governments of Cameroon and Nigeria. A joint commission has been formed by the two governments to investigate the dam and to make recommendations for mitigation of the hazard. Results of ongoing engineering studies of the dam were reported by commission members at a special workshop on Lake Nyos held in Lagos, Nigeria, on 5 December 1990; the joint commission will present a preliminary report of these investigations to appropriate agencies early in 1991. The dam threat was also discussed at the Symposium on the Lake Nyos Disaster, at the 15th Colloquium on African Geology held in Nancy, France, in September 1990, but these discussions were not mentioned in the published meeting report⁴.

Concern was raised at the Nancy meeting that lowering the lake level to mitigate the

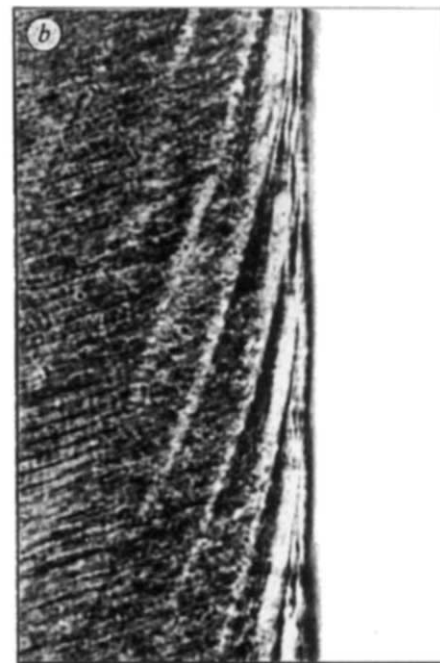
natural dam hazard could cause a massive release of lethal CO₂ from the lake waters or underlying sediments. We recognize this hazard and feel that additional study of the factors controlling gas accumulation and release is needed. But our inspection of the dam has convinced us that it is not safe and that there is a strong possibility of failure within the not-too-distant future. The potential loss of life from failure of the dam is much greater than that from another gas release because of the current downstream population distribution. Measures to warn threatened populations are urgently needed. Action to remove the flooding hazard by lowering the lake level should proceed as soon as engineering studies provide viable options, including a method of controlled degassing of CO₂ from the lake.

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On the surface — demarcating perikymata (a) and smooth enamel surface (b).

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Hominid dental development

SIR — Mann, Monge and Lampl have disputed¹ the findings of two previous studies^{2,3} that claim a short period of dental development in hominids, but they misunderstand the arguments regarding tooth development. These new studies rely on endogenous circadian and circaseptan rhythms that operate during tooth development. Perikymata are secondary manifestations, on the tooth surface, of an internal 7–9 day cyclical variation in enamel secretion, which produces striae. Secretion slows at the enamel surface, either abruptly with striae terminating in troughs (demarcating perikymata, part a of figure), or progressively, resulting in a smooth enamel surface (see part b of figure). It is unnecessary to invoke “abrasive phenomena”¹ to account for the absence of perikymata.

A. D. B. and D. Reid (manuscript in preparation) have made ground sections of 31 unworn modern human incisor teeth, selected for perikymata expression where possible, allowing counts to be made in imbricational enamel of surface striae ($\pm$ s.d.) in (n_s) teeth, and range, together with perikymata counts ($\pm$ s.d.) in (n_p) teeth:

I₁: (10) 132 $\pm$ 9.9, 111–154; (7) 133 $\pm$ 15.5

I₂: (6) 141 $\pm$ 20.0, 110–164; (4) 146 $\pm$ 10.0

I¹: (7) 160 $\pm$ 26.0, 135–208; (4) >74

I²: (8) 139 $\pm$ 11.3, 123–153; (7) 140 $\pm$ 10.6

Imbricational striae counts corresponded closely to perikymata counts in I₁, I₂, and I² and counts decreased in the series I¹ > I₂, > I² > I₁.

Perikymata are variably expressed, and counts are reliable only in teeth showing a continuous series. I¹ showed least consistency in perikymata expression, with no tooth showing a complete perikymata series from cervix to incisal edge. These results suggest that Mann *et al.*'s I¹ specimen is not expressing all of its subsurface striae, and should be excluded from further consideration. To establish the minimum chronological age of this individual, the tooth (I₁) with highest perikymata counts (157) must be used. Crown formation time is 3.5–3.9 years (7–8 day periodicity and 0.5 yr buried increments). Age at death includes an interval between birth to onset of mineralization (estimated 0.25 yr), plus time to form 3 mm of root at a rate of 3.8–2.9 (ref. 4) μ m day⁻¹ (2.2–2.8 yr) giving an age at death of between 6 and 7 yr. This estimate is wholly consistent with the eruption status of M1, which erupts between 6 and 7 yr in modern humans (against 2.2–3.7 yr)¹.

We are acutely aware of the inconstant expression of perikymata, having studied all the several hundred available hominid teeth from Koobi Fora, Kenya; Olduvai Gorge, Tanzania and Sterkfontein, Kromdraai and Swartkrans in South Africa. Incisors without complete perikymata expression were excluded, leaving only nine *Paranthropus*, five *Australopithecus*, and four early *Homo* teeth. Mann *et al.*¹ do not acknowledge differences in perikymata counts between gracile and robust australopithecines^{2,3}. Mean *Paranthropus* (SK62 R and L; SK63; SK71; SK73; OH30; ER812; ER1477; ER1820) I₁ perikymata counts³ ($n=9$), 81 $\pm$ 14.3, are highly significantly smaller ($P<0.0001$)

than our modern human I₁ sample cited above; and also Mann *et al.*'s¹ combined incisor sample which includes low counts ($n=12$), 116 $\pm$ 25 ($P<0.004$). *Australopithecus* (Sts24a; LH2; LH3 I¹ I²; LH6) combined incisor³ counts ($n=5$), 146.2 $\pm$ 24.5 are not significantly different from either modern human sample.

Apes have large teeth which take longer to form than modern man, yet erupt earlier, achieved by more rapid rates of root development⁵. Foreshortened growth periods in *Australopithecus* seem to be linked with faster root growth³. Smith has shown⁶ that the age of M1 eruption in primates is highly correlated [$r=0.98$] with adult brain weight, suggesting ape-like M1 eruption times in early hominids. Both microanatomical and comparative evidence agree in suggesting rapid tooth formation, early eruption of teeth and short periods of growth and development in early hominids.

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■ This correspondence is now closed — Editor, Scientific Correspondence.

Telling tails

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Physics and Chemistry of Comets. Edited by W. F. Heubner. Springer: 1990. Pp. 392. £35, \$59.

SMALL, frigid, charcoal-black bodies composed of ice and dust, comets are as enigmatic as they are remote. Normally too distant for detailed study, they are observed only during relatively close passage to the Sun, where heat-liberated gas and dust forms a vast coma and tail complex, the largest visible structures in the Solar System. Solar-induced activity unfortunately also obscures the nucleus and, until recently, the true nature of the actual cometary body could only be inferred from observed coma and tail properties. An enormous increase in information on comets occurred in 1986 when five spacecraft from Europe, the Soviet Union and Japan encountered comet Halley on its thirtieth known passage through the inner Solar System. Some of these fly-by missions provided the first direct images of a comet nucleus and they penetrated deep into the coma where direct *in situ* measurements were made of dust and gaseous species. These mission data, along with results from Earth-based and even Venus-based observations and generally intensified attention to comets produced a *bona fide* quantum leap in our knowledge about them.

The general premise that comets are primitive, irregular bodies predominantly composed of water, ice and silicate-bearing dust was confirmed, at least for Halley, although in many ways it now appears that comets are more complex and even more interesting than previously believed. Complications include bi-axial rotation, a largely inert surface that is only active in localized regions, coma grains that fragment in space, components that are volatile when as far from the Sun as Saturn, a possible mass dominance of rock over ice, a 'depletion' of carbon and nitrogen in ice phases, and the presence of material intermediate in volatility between ice and dust that decomposes in the coma to produce a distributed source of carbon monoxide, formaldehyde and other compounds. Although there is a new realization that the pristine nature of comets has been somewhat degraded by thermal processes associated with internal heating and encounters with the Sun and other stars, most of the new results and modelling studies support the general belief that comets are the best preserved

relics from the formation of the Solar System and that they preserve materials and precious records of chemical and physical processes that occurred in the cold regions of the solar nebula and possibly even in environments that preceded it.

There is a substantial body of recent

IMAGE
UNAVAILABLE
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REASONS

Comet Giacobini-Zinner — released dust has formed two tails.

literature on comets, but gaining a balanced overview of the subject is difficult even for those specializing in this interdisciplinary field. Several problems complicate the synthesis of comet data. One is the traditional problem of temporally fragmented observations of highly variable objects. Special limitations of the Halley *in situ* results are a consequence of data collection from a spacecraft travelling at 70 kilometres per second during a close fly-by of a ten-kilometre object. With the need for synthesis in mind, *Physics and Chemistry of Comets* is a very timely book. The time delay following the

Halley encounters has allowed the data to be properly reduced, analysed, contemplated and cross-correlated. The resulting book provides a superlative overview of comets and cometary processes. The editor and ten well-chosen experts covering the breadth of cometary disciplines have produced a wonderfully comprehensive and yet concise overview of cometary physics and chemistry.

Each chapter is a review paper that provides a reasonably balanced view of its subject. The coverage and continuity is very good and is evidence of careful editorial direction to minimize overlaps and to broaden the coverage to include some information on various cometary subjects that are of only peripheral importance to the physics and chemistry. The main chapters cover the nucleus, plasma, the neutral coma, dust, orbital evolution and comet formation and evolution. The final chapter is an overview, plus a discussion of the role of comets in the evolution of life and a discussion of major problems and directions of future research. This is not a compendium of a large number of research papers, and there are aspects of cometary research that are only lightly treated, but the great value of the book is that it provides a concise and detailed review of the major issues related to the chemistry and physics of comets. Unlike many other modern works on Solar System topics, this one is a slim, concisely crafted reference that easily fits in a briefcase.

With its excellent reviews, a chapter by J. H. Oort on the Oort cloud and a preface by F. L. Whipple this book is essential for those working on comets, and with 776 references it is a highly recommended resource for anyone who needs up-to-date information on the fundamental aspects of comets. Many of the chapters contain summaries that can provide even a casual reader with a quick review of each subject. Comets are unique objects that provide a potential link between studies of primitive materials of the Solar System and pre-solar matter.

Although many of the most important properties of comets remain unexplored and elusive, *Physics and Chemistry of Comets* provides an important resource for the next generation of comet studies, which will hopefully include new observational methods and spacecraft missions that will both rendezvous with a nucleus and return samples to the Earth for laboratory analysis. □

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Growing old gracefully

Linda Partridge

Longevity, Senescence and the Genome. By Caleb E. Finch. *University of Chicago Press*: 1991. Pp. 922. \$49.95, £39.95.

THE word 'ageing' has different implications for wine and women. In *Longevity, Senescence and the Genome* Caleb Finch reacts to this diversity of meaning by banning the word from his book, preferring instead to confine himself to 'senescence'. As Finch points out, things can get better as well as worse during the course of life; very young animals are often more likely to die than those at the peak of their powers, and animals that grow as adults often become more reproductively successful as they do so. Finch has sown the seeds of some confusion by making the tacit assumption that similar mechanisms are responsible for these two kinds of change.

Although usages differ in important detail, gerontologists and evolutionary biologists both use the term 'ageing' in a general way to refer to the drop in biological performance late in life. Ever since the scientific study of ageing started with August Weismann, there has been a gulf between those working on the proximate mechanisms responsible and those interested in the ultimate evolutionary reasons for its existence. Finch makes a concerted effort to unite the two approaches, which is a thoroughly worthwhile undertaking, and he has drawn together a large and scattered literature on ageing, and looked for some generalizations. Few emerge, partly because the integration of gerontological and evolutionary approaches is incomplete.

Evolutionary biologists think they understand different kinds of age-related changes, at least in broad outline. Some events, such as those of embryonic development and the onset of reproduction, are the products of

natural selection, and are controlled by mechanisms ensuring that they occur at appropriate times in the life history. Senescence is not like development: there is no predictable set of events that occurs at similar times in all members of a species. Rather, almost anything that can go wrong does go wrong in at least some cases, and the timing and extent of decline is enormously variable. The haphazard nature of senescence can be explained if it is not the product of natural selection, and is in fact maladaptive. It can none the less evolve for two reasons. First, natural selection may fail to eliminate mutations that impair survival or reproduction in old age, because most of the carriers will have died of something else by then anyway. Second, there may be bad, but weakly selected, side-effects late in life of mutations that are advantageous earlier on. Either way, ageing is expected to evolve and to decrease the likelihood of both survival and reproduction later in life.

The rate of ageing that evolves will depend upon demography in nature. A risky way of life will produce high, externally imposed, age-independent mortality rates, and will lead to the evolution of rapid senescence. One of the unique strengths of the book is that changes in the rate of ageing are clearly distinguished from changes in death rate unrelated to age, and the former do indeed vary enormously between species: Finch quotes mortality rate doubling times of 27 years for river perch, eight years for elephants, six months for white-footed mice and one week for *Drosophila melanogaster*. He does not examine the association between hazard and the rate of senescence, which would have been interesting.

Against this theoretical background, some of the questions Finch poses do not make a lot of sense. For instance, he frequently refers to the idea that senescence is controlled by one or more pacemakers, which might reside in the genome. He therefore presents a review of the ways in which gene expression and fidelity of DNA replication change during the lifespan of well-studied organisms. This information is comprehensive, well presented and very interesting. But the attempt to find the top of a hierarchy of controls for ageing is likely to prove profitless if many processes go wrong simultaneously, as evolutionary theories predict.

We need to understand the details of what goes wrong during ageing if we are to stand any chance of informed medical intervention. This kind of approach has occupied countless gerontologists and has produced an almost unmanageably large amount of information. One prediction of evolutionary theories of ageing is that it is likely to be affected by many genes and to involve simultaneous decline in those processes responsible for continuing survival and fertility. As the data marshalled by Finch clearly demonstrate, the overall picture is confusing. Elephants loose their teeth whereas male marsupial mice *Antechinus* die from the side

effects of the hormones responsible for suicidal rates of reproduction. The evolutionary approach would have something to offer in explaining this diversity. Most mechanistic theories of ageing are theories of damage through release of free radicals, somatic mutation, errors in protein synthesis, injury to joints and so on. Which of these particular hazards is most pressing will depend upon the way of life of the organism concerned. It must be presumed that when rates of ageing evolve, they do so through alteration in the extent to which at least some of these forms of damage are combatted. The interesting questions then become comparative: do bats repair their DNA more than small rodents and do storm petrels mop up free radicals more effectively than starlings? These are, of course, difficult questions to answer.

Finch takes a slightly different tack, and attempts to test the evolutionary theories by a detailed examination of what goes wrong in particular systems. The data are again interesting, but not obviously relevant. For instance, the fact that the ageing pigments lipofuscins accumulate at different rates in various cell types in mice does not seem to contradict the theory, as Finch suggests. What matters is how these different rates of change affect survival and reproduction. When Henry Ford inspected the scrap heaps to find out what economies in manufacture could be made, he was not interested in the numbers of scratches on the steering wheels and the cylinder heads.

An oddity of the text, although one with a distinguished pedigree in gerontology, is the lack of emphasis on the decline in fertility later in life. From the evolutionary point of view of genetic contribution to the next generation, this is as serious as the decline in survival, and the mechanisms producing it are of as much interest as those impairing survival. Finch claims that reproductive determinism, in other words a fixed quota of germ cells, is the only mechanism that would produce a decline in fertility with age. This seems an unlikely explanation in many cases, because individuals that are prevented from using germ cells early in life still show the later decline. One interesting point to emerge from the discussions of reproduction is that semelparity (breeding only once) does seem to be an evolutionary black hole from which repeated breeding does not re-evolve.

An enormous virtue of Finch's text is that it does not confine itself to the traditional model organisms for the study of ageing: the fruitfly, the nematode and the mouse. This is a treasure house of fascinating information about other organisms, which will undoubtedly pave the way for comparative work. □

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NEW JOURNALS ISSUE

This year, *Nature's* annual new journals review supplement will appear in the issue of 3 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals that first appeared after June 1989 and issued at least four separate numbers by the end of April 1991 will be considered.
- The deadline for submission is end of May.
- Journals covering any aspect of science are eligible, but those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- The journals must be published at least three times a year.
- The main language used must be English.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others).

A hero's fight

Barbara J. Culliton

Beyond Love. By Dominique Lapierre. UK Century/US Warner: 1991. Pp.464. £15/\$22.95.

BEYOND LOVE is the only book I have read that carries a dust-jacket endorsement from the Pope. John Paul II called it "a remarkable testimony of human solidarity".

And indeed it is. This is on one level a simple, even pat, tale of heroes who fight disease and, when defeated, bravely face death. Written by popular French author Dominique Lapierre, *Beyond Love* weaves the story of the AIDS epidemic with the saga of a young Indian untouchable who literally emerges from the river Ganges, where her low-caste family over-sees the burial of the dead, to become a shining light in Mother Teresa's Palace of the Immaculate Heart where her nuns ministered to the dying poor.

Lapierre, who spends a good deal of his time in India, also spent time interviewing American and French AIDS researchers for his account of AIDS, which begins dramatically in room 516 of the hospital at the University of California at Los Angeles. Here, a freelance homosexual model with bizarre symptoms is seen by Michael Gottlieb, a dedicated young immunologist with "a fertile mind" who leaves no book unread in his effort to figure out why his 31 year-old patient has lost his immune system. It was Gottlieb, not much mentioned any longer in the AIDS drama, who saw the world's first diagnosed AIDS patients in 1980.

Lapierre traces the course of AIDS through medical circles as physicians in New York and elsewhere discovered that they all had as patients young homosexual men who were dying of a strange immune disorder. From there it is a quick leap to the Centers for Disease Control in Atlanta where in 1981 a "commando of very special supercops" headed by James Curran faces the fact that a new disease of potential epidemic proportions has been unleashed on the world.

No tale of AIDS would be complete without Robert C. Gallo of the US National Cancer Institute and Luc Montagnier of the Institut Pasteur in Paris, and Lapierre does not disappoint us. Lapierre takes the French view of argued events, casting Gallo as Goliath and Montagnier as David. By setting the most dramatic research scenes of his soap opera at the Pasteur, he portrays Montagnier, his former colleague Jean Claude-Chermann and Francoise Barre-Sinoussi as

the basic scientist heroes of the AIDS campaign. In keeping with the stereotypical character of the book, Lapierre falls into the trap of describing the men in terms of experiences that affected their lives as scientists while expressing implicit amazement that a woman can be a woman and a researcher at the same time. Thus, we learn that Barre-Sinoussi was "just as capable of rustling up a traditional blanquette of veal or a Grand Marnier soufflé as she was of lovingly cultivating her fragile lymphocytes." How about that.

A sophisticated reader will find Lapierre's language and style that of a grade-B novel. Biologists coming to this book will find errors. It is not a prize-worthy piece of writing.

Nevertheless, *Beyond Love* is in its way a

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Mother Teresa — interleaved with chapters on AIDS.

good job for a book meant to have mass appeal, largely because it does convey overall the drama, the ups and downs, and the competition that are part of the battle against AIDS. And if its pattern of interleaving chapters about AIDS with chapters about the unwashed of India is a heavy-handed way of saying AIDS patients in the West are treated like lepers, the story of Mother Teresa's innocent and devoted nuns is still moving in parts.

One aspect of AIDS that Lapierre handles deftly is the agony of death. So much press attention has been focused on the research competition that the clinical picture is obscured. Not so in *Beyond Love*, which includes this account of the impending death of a fictional man named Josef Stein who is "giving up the fight". "The effect of surrender was immediate: in the hours that followed Josef Stein was struck by a fresh attack of pneumocystis pneumonia. Each fit of coughing seemed likely to deliver the last blow. The lesions of Kaposi's sarcoma had reached his salivary glands, burning his tongue and throat with a burst of fire no

liquid could quench." Stein survived a few days until the violet Kaposi's pustules "blocked the entrance to the esophagus altogether," and he died gasping for air.

It is not pleasant reading, but it is part of the real story of AIDS and is something Lapierre rightly includes. This is a book that is hard to classify, being at once simplistic, even trite in language and structure, and yet nonetheless moving overall. If one forgets the details and focuses only on the general impression a lay reader will get from this book, *Beyond Love* does an interesting job of informing the public about some of the realities of science and medicine. □

Barbara J. Culliton is Deputy Editor of Nature.

Sketches of science

James C. G. Walker

Chemical Evolution: Origin of the Elements, Molecules and Living Systems. By Stephen F. Mason. Oxford University Press. 1991. Pp.317. £19.50.

AMONG the presents described by Dylan Thomas in *A Child's Christmas in Wales* were "books that told me everything about the wasp, except why". *Chemical Evolution* by Stephen F. Mason is one of those books, not about the wasp of course, but about the origin and evolution of nearly everything.

The idea of telling in one book the story of the origin of the elements, stars, galaxies, planets and life is not new. Much the same ground was covered by Wallace S. Broecker in *How to Build a Habitable Planet* (Eldigio, 1985). What is new here is the combination of the history of matter in the Universe with the history of the scientific study of this matter. Together, these add up to a lot of history. The subjects are all here, from 'absolute magnitude' to 'zinc', 'zircon' and 'zymase'. So are the people, from Adams, W. S. (1876–1956) to Zeeman, P. (1865–1943). Apart from Hipparchus of Rhodes (c.170–c.125 bc), whose observation of a solar eclipse in 129 bc has been used to measure the lengthening of the day, the oldest birth dates in the index are those of W. Gilbert (1544–1603), T. Brahe (1546–1601), J. Kepler (1571–1630) and J. B. van Helmont (c.1579–1644). Gilbert studied terrestrial magnetism at the court of Queen Elizabeth, and van Helmont worked in Brussels on the transmutation of elements. The most recent birth date seems to be that of R. W. Wilson (1936), who discovered, with A. A. Penzias (1933), the cosmic microwave background radiation.

Regrettably, it is more fun to dip into the index for small facts about scientific notables than it is actually to read the book. The author describes his work as being in the

Mark Edwards/Still Pictures

"historico-encyclopaedist tradition", and that is a reasonable characterization. The information is here, but barely. Kepler, for example, rates a sentence on page 64, in reference to his study of the nova of 1604, and on page 85, a mention of the same study. Priestley, perhaps more central to the history of chemistry than Kepler, gets one sentence on page 130 and two sentences on page 162, no more. So we cannot look here for illumination on what made the great scientists work, where they got their ideas, or what prejudices coloured their work. Why, for example, was Lord Kelvin unwilling to accept a great age for the Earth? Was it a religious bias, conscious or unconscious, or did he have a low opinion of geologists? Why did a brilliant geophysicist like Sir Harold Jeffreys totally fail to imagine any way that continents could drift? Was his opposition to drift a consequence of some long ago squabble in the common room or a deeply felt faith in an unchanging Earth?

Questions such as these should be the stuff of science history, not a catalogue of ideas and their contributors. We gain a better understanding of the science of our day and its likely limitations from a consideration of the social and cultural factors that caused spectacular errors by the giants on whose shoulders we stand. This attitude to history, exemplified by much of the writing of Stephen Jay Gould, calls for attention to historical detail, detail that the reader will not find in this book.

On the other hand, the 'historico-encyclopaedic tradition', in this manifestation, fails also to describe the origin of elements, molecules and living systems in sufficient detail to educate readers not already familiar with the science. A journey from 'the chemical elements in nineteenth-century science' to 'biomolecular handedness' by way of nucleosynthesis, origins of planetary systems, chemiosmosis and prebiotic chemistry is too long for 284 pages of text, when these pages must present history as well as science. The result is indeed an encyclopaedia, offering thumbnail sketches of important results and important scientists, not a book from which to learn science or history.

Furthermore, this is not an easy book to read. Admittedly, much of the material is difficult, but the language is also unnecessarily difficult. In the last paragraph of the introduction, a paragraph that is in no way special, the average number of syllables per word is two. For comparison, the last paragraph of the first chapter of Gould's book, *Wonderful Life: The Burgess Shale and the Nature of History* (Norton, 1989), another technical treatment of difficult science history, has on average 1.35 syllables per word. Which paragraph is more fun to read? □

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Memory's Holy Grail

Stuart Sutherland

In The Palaces of Memory: How We Build The Worlds Inside Our Heads. By George Johnson. Knopf: 1991. Pp.255. \$22.95.

WITH the same zeal that mediaeval knights devoted to the search for the Holy Grail, neuroscientists have been seeking the engram, the physiological change underlying memory. The comparison is not inapt. Just as the discovery of the cup from which Jesus sipped would not alter Christian doctrine, so the discovery of the engram would scarcely change the way we think about memory: knowing how a transistor works is irrelevant to understanding computer programs, and it is how memory is programmed that is of most interest. Moreover, according to George Johnson's account, the jousting between the scientists bent on the quest for the engram has been at least as formidable and rather more acrimonious than that in which ancient knights participated. Finally, both quests have met with a marked lack of success.

In *The Palaces of Memory* records scientific discoveries by concentrating on the careers of one or two scientists; it intersperses accounts of their work with reasonably entertaining gossip. The protagonist of the first half of the book is Gary Lynch, a rebel who "hated school" and who rather than attending talks at conferences would hold "drinkathlons" with his acolytes. On a rather flimsy empirical basis, he speculated that when a neuron is fired, calcium channels open, the calcium that pours into the cell activates a substance called calpain, which attacks the cell's cytoskeleton, a structure that supports the cell membrane. Finally, the weakened membrane allows hidden and therefore inactive receptors to pop through: their presence on the surface of the dendrites makes the cell more receptive to stimulation. Lynch attempted to support this ingenious story by a series of no less ingenious but largely inconclusive experiments. His theoretical edifice eventually collapsed when it was shown in another laboratory that he had misinterpreted one of his most crucial experiments: he had mistakenly concluded that at synapses where learning was assumed to have occurred there was an increase in the amount of neurotransmitter binding to receptors (which would of course occur if the learning mechanism was determined by an increase in receptors). Lynch modified his theory and lived to fight another day.

Johnson's account of Lynch and his work is gripping. It gives the layman and scientists in other disciplines an idea of the almost incredible complexity of the nerve cell and synapse, and of the vast number of biochemicals involved. By comparison, the transistor

is child's play. One wonders what the function of such complexity can be and marvels that when there is so much to go wrong, the mechanisms are for the most part so reliable.

Despite the fascination of the story of Lynch (which I have greatly truncated), looking at a scientific field through the eyes of one man can be misleading. Many other scientists have been seeking the engram, and their work may prove at least as important as Lynch's; Johnson either mentions them rather casually or ignores them altogether. The one exception is Eric Kandel, of whom Johnson remarks, "some of his colleagues attributed his success less to the merits of his theories than to his abilities at intellectual salesmanship". Kandel worked on the sea slug, a lowly beast with only about 20,000 neurons in its central nervous system. He proposed a model of the engram in which the change is in the presynaptic neuron not, as Lynch suggested, the postsynaptic one. There are now several different theories of the engram, none of which is satisfactory. Moreover, there is no reason to believe that it will turn out to be the same in all phyla or even in all parts of the human brain.

Johnson goes on to discuss neural nets, basing much of his account on Leon Cooper, who although one of the first in the field has hardly been the most influential. He stresses the antagonism between artificial intelligence (AI) and connectionism, but apparently this has been resolved by Marvin Minsky (one of the founders of AI and according to Johnson "famous for his sarcasm"), who performed the unaccustomed role of a diplomat at a connectionist conference. Many, but not all, now accept that in the hierarchy of the brain, AI rules at a high level, neural nets at lower ones.

Johnson ends with a brief discussion of the philosophy of science and of mind. His heroine for the last act is, for no apparent reason Patricia Churchland, an extreme reductionist. Few of the counter arguments are given and consciousness is not mentioned.

I may be biased, but it seems odd that almost no psychological research on memory is mentioned. Psychologists have after all established that there are three different kinds of memory, which last for very different times. Moreover, even rats can connect in their memory events that are separated by a four-hour time gap. These and many other facts pose a challenge for physiological theories of the engram. Nevertheless, Johnson has written a fascinating book, which perhaps throws as much light on how science is done and on the scientists who do it as any book since *The Double Helix*: all it lacks is a denouement. But that would be hard to provide, because while the knights of old inhabited real castles, most workers on memory have built castles in the air. □

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Dynamic melting of the Iceland plume

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Icelandic high-magnesia basalts show striking correlations between major element abundances and incompatible element and radiogenic isotope ratios. The most MgO-rich lavas have the most depleted incompatible element ratios and among the least radiogenic lead isotopes recorded in Atlantic mid-ocean-ridge basalts, highlighting a decoupling of the major and trace element characteristics expected of plume melts. This paradox can be explained by the process that mixes melts segregated from different depths of the melting column. The resulting model provides insight into the processes governing melt compositions at spreading ridges.

ADVANCES in the understanding of the physics of partial melting¹ have made possible the quantitative modelling of mantle melt production during extension². The volume of melt produced depends on the amount of extension and the temperature of the mantle induced to upwell during extension. The relationship between these parameters is most readily quantified for mid-ocean rifting, as the stretching factor is infinite, and the volume of melt produced is manifest in the thickness of oceanic crust. Thus oceanic crust thickness should depend principally on the temperature of the underlying, upwelling mantle. Moreover, as melt composition is dependent on the degree of melting and thus on the volume of melt produced beneath any rift, there should be a relationship between the thickness of the oceanic crust and its chemistry, as observed by Klein and Langmuir³.

The thickest segment of actively rifting oceanic crust is beneath Iceland, formed as a result of high degrees of melting of mantle some 200 °C hotter than that which upwells beneath 'normal' ocean ridges. As high-degree melts are MgO-rich⁴⁻⁷, high MgO contents should be characteristic of lavas erupted on Iceland.

The mantle source beneath Iceland is, however, not only hotter, but also geochemically 'enriched' in comparison with the mantle beneath normal spreading ridges (N-MORB source). Numerous studies have shown Icelandic basalts to have more radiogenic lead and strontium isotope ratios, less radiogenic ¹⁴³Nd/¹⁴⁴Nd ratios, and incompatible-element ratios reflecting higher concentrations of the more incompatible element (for example, higher La/Sm and Rb/K), with respect to Atlantic mid-ocean-ridge basalt (MORB)⁸⁻¹³. Primitive melts from the thermally and geochemically anomalous mantle of the Icelandic plume are therefore expected both to be MgO rich and to have enriched trace-element and isotope signatures compared with MORB.

Here we present new major element, trace element and radiogenic isotope data on two high-MgO lava suites from Iceland. Intriguingly, both of these high-MgO suites show depleted geochemical signatures with respect to typical, lower-MgO Icelandic tholeiites. Furthermore, within each suite, the most MgO-rich lavas show the most depleted signatures, and the lead isotope data include some of the most unradiogenic values measured in Atlantic MORB. The model developed to explain this striking decoupling of the predicted major- and incompat-

ible-element characteristics expected of plume melts has important implications for partial melting processes along mid-ocean ridges.

The high-MgO lavas

The two sampling locations, the Reykjanes Peninsula and Theistareykir, are at the southwest and northeast extremities of the central Iceland rift and represent onshore continuations of the Reykjanes and Kolbeinsey ridge sections of the Mid-Atlantic Rift respectively (Fig. 1). We analysed whole rock samples of crystalline lavas that are all post-glacial (<10,000 yr) and very fresh. Table 1 presents selected data and summarizes the analytical techniques used. The lavas studied are picrites (with olivine, 88–91 mol% forsterite, and chrome spinel phenocrysts) and olivine tholeiites (which can be aphyric, olivine, plagioclase or olivine/plagioclase phytic). The very close spatial and temporal distribution of the samples in each suite minimizes any changes in source over space or time, and thus suggests that the geochemical variations are largely process controlled.

There is a large range in MgO content, 8–19% (Mg# = $Mg^{2+}/(Mg^{2+} + Fe^{2+}) = 58–82$, Table 1), which correlates inversely with the contents of SiO₂ and FeO (Fig. 2a and b). The effects of crystal fractionation for such high-MgO tholeiitic compositions are quite easy to assess, as only olivine (plus

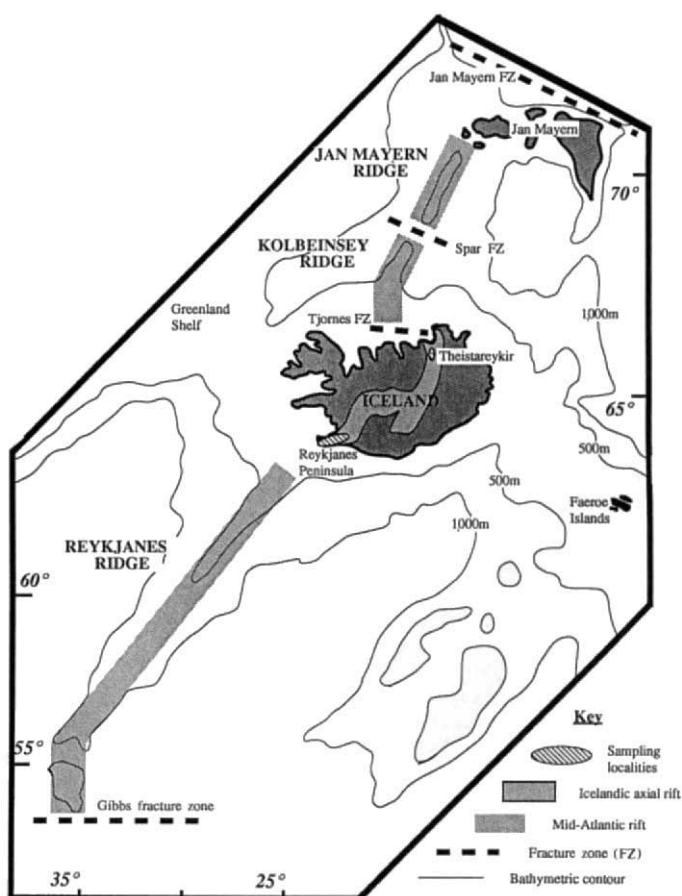


FIG. 1 Bathymetry and location of Iceland, sampling localities and adjacent segments of the Mid-Atlantic Ridge.

TABLE 1 Selected geographical, chemical and petrographic data for the high-MgO basalts

Sample	Suite	Latitude	Longitude	Modal phenocryst content	MgO	FeO	Mg#	K ₂ O	¹⁴³ Nd/ ¹⁴⁴ Nd	⁸⁷ Sr/ ⁸⁶ Sr	²⁰⁶ Pb/ ²⁰⁴ Pb	²⁰⁷ Pb/ ²⁰⁴ Pb	²⁰⁸ Pb/ ²⁰⁴ Pb
D2/11	R	63° 48' N	22° 39' W	20% Ol (+Cr Sp.)	19.64	7.46	82	0.006	0.513158		18.197	15.401	37.741
D6	R	63° 51' N	22° 34' W	Aphyric	10.30	8.98	67	0.102			18.556	15.412	37.984
D7	R	63° 53' N	22° 32' W	8% Ol (+Cr Sp.)	12.88	7.39	76	0.009			18.268	15.432	37.859
D8A	R	63° 52' N	22° 23' W	10% Ol, 3% Plag	9.30	9.64	63	0.093	0.513054	0.703188 ± 16	18.525	15.453	38.035
D8B	R	63° 53' N	22° 23' W	8% Ol (+Cr Sp.), 4% Plag	14.62	7.69	77	0.031	0.513103		18.422	15.460	38.005
D18	R	63° 52' N	21° 42' W	5% Ol (+Cr Sp.)	11.73	9.11	70	0.040	0.513093		18.298	15.439	37.957
D26	R	63° 54' N	21° 28' W	5% Ol (+Cr Sp.)	11.21	8.42	70	0.033			18.411	15.436	37.959
D27	R	63° 55' N	21° 24' W	5% Ol (+Cr Sp.)	13.08	7.57	75	0.017			18.323	15.439	37.862
IT1	T	65° 53' N	17° 09' W	2% Plag	9.19	8.96	65	0.061			18.058	15.417	37.685
IT2	T	65° 53' N	17° 03' W	4% Plag	8.07	9.77	60	0.113	0.513070	0.703225 ± 8	18.035	15.374	37.574
IT3A	T	65° 49' N	16° 54' W	3% Ol, 3% Plag	9.07	9.86	62	0.127		0.703238 ± 12	18.060	15.410	37.704
IT4	T	65° 50' N	16° 50' W	2% Ol, 2% Plag	7.91	10.24	58	0.144	0.512997	0.703071 ± 10	18.005	15.335	37.475
IT5	T	65° 52' N	17° 03' W	20% Ol (+Cr Sp.)	15.84	8.92	76	0.035	0.513105	0.703044 ± 10	17.980	15.352	37.441
IT6	T	65° 52' N	17° 03' W	4% Ol (+Cr Sp.)	10.60	8.65	69	0.045	0.513090		17.983	15.367	37.491
IT7	T	65° 48' N	16° 58' W	4% Ol (+Cr Sp.)	11.45	8.30	71	0.053	0.513098		17.988	15.380	37.518
TH8	T	65° 57' N	17° 05' W	5% Ol (+Cr Sp.)	12.40	8.46	72	0.033	0.513128		17.957	15.363	37.452
TH13	T	65° 54' N	16° 52' W	4% Ol (+Cr Sp.)	9.78	9.24	65	0.055	0.513113	0.703020 ± 20	18.064	15.399	37.643
TH29	T	65° 55' N	17° 04' W	15% Ol (+Cr Sp.)	15.73	7.39	79	0.011	0.513155	0.702928 ± 16	17.800	15.344	37.263

R indicates samples from the Reykjanes Peninsula and T indicates samples from Theistareykir. Major elements were analysed by X-ray fluorescence (XRF) on fused pellets with a heavy lanthanum absorber. The FeO content was calculated from total Fe₂O₃ assuming Fe³⁺/(Fe²⁺ + Fe³⁺) = 0.1. Neodymium was run as metal with ¹⁴³Nd/¹⁴⁴Nd normalized to ¹⁴⁶Nd/¹⁴⁴Nd = 0.72190 within run, and the final ¹⁴³Nd/¹⁴⁴Nd renormalized to ¹⁴²Nd/¹⁴⁴Nd = 1.142025, using the mean, Ce-corrected ¹⁴²Nd/¹⁴⁴Nd of the sample. The ratio ⁸⁷Sr/⁸⁶Sr was normalized to ⁸⁶Sr/⁸⁸Sr = 0.1194. Errors quoted are 2σ/√N statistics for each run, and replicate standard runs indicate a reproducibility of 0.007‰ for ¹⁴³Nd/¹⁴⁴Nd (2σ on 4 runs) and 0.005‰ for ⁸⁷Sr/⁸⁶Sr (2σ on 3 runs). These techniques give ¹⁴³Nd/¹⁴⁴Nd = 0.512638 for BCR-1 and 0.710329 for NBS 987. Blanks are less than 2 ng and 0.3 ng for Sr and Nd respectively, and thus insignificant. Lead was run at a controlled temperature of 1,200 °C using an adaptation of the silica-gel technique of Manhès³⁹. Reproducibility of standards and samples is better than 0.04‰ per AMU (2σ on 8 runs of NBS 981). Lead isotope ratios have been corrected using a fractionation factor of -0.06‰ as indicated from the mean values obtained for NBS 981. Lead blanks vary from 0.1 to 0.2 ng. They represent <0.5% of total sample load for the lowest-lead (low-FeO) samples, and so are insignificant.

chrome spinel) is expected to fractionate until plagioclase becomes cotectic^{14,15} at Mg# ≈ 65. Vectors for 15% olivine fractionation are shown in Fig. 2, and it is clear that this can account for increasing SiO₂ with decreasing MgO (Fig. 2a). Similarly, addition of olivine can explain the departure of the two aberrant points from the main trend in Fig. 2b, which is also consistent with the abundant olivine phenocrysts present in these two samples (Table 1). But to generate the main negative FeO–MgO trend in Fig. 2b by fractional crystallization would require plagioclase to be cotectic with olivine for the whole range of lava compositions, which is implausible. Furthermore, such fractionation of olivine and plagioclase would cause an increase in CaO/Al₂O₃ with increasing FeO. Although the trend is scattered, the opposite is observed (Fig. 2c). Thus variations in FeO do not seem to be a result of crystal fractionation, and FeO may be used as an index of primary major-element composition.

The content of FeO correlates with both incompatible-element contents and incompatible-element ratios (Fig. 2d and e). The variations of incompatible-element contents in the high-MgO lavas are very large (K₂O contents vary by over an order of magnitude, Fig. 3a), and are greatest for elements with the lowest inferred distribution coefficients for melts in equilibrium with a mantle source¹⁶. Thus the lowest-FeO (highest-MgO) lavas have the lowest incompatible-element concentrations and the most depleted incompatible-element ratios. This is unexpected, because if the highest-MgO melts are most representative of high-degree melts from the plume, they should have enriched incompatible-element ratios, characteristic of the plume source.

If the lower-FeO (higher-MgO) melts are thought to represent higher-degree melts, they would be expected to have lower incompatible-element contents. But the variations in incompatible element contents between the highest- and lowest-FeO lavas of this study are greater than could reasonably be explained by differences in degrees of partial melting using simple melting models¹⁷, and additionally, differences in degrees of melting for such high-degree tholeiitic melts are unlikely to fractionate incompatible trace element ratios significantly.

Previous studies have noted that incompatible element ratios vary with both ⁸⁷Sr/⁸⁶Sr and ¹⁴³Nd/¹⁴⁴Nd for Icelandic basalts^{18–20}. This study additionally reveals correlations of incompatible element and isotope ratios with major components such as FeO (Fig. 4). Although the two suites have similar Nd but distinct Pb isotope ratios, it is clear from Fig. 4a and b that the lowest-FeO (highest-MgO) lavas of each suite have the least

radiogenic ²⁰⁶Pb/²⁰⁴Pb, and the most radiogenic ¹⁴³Nd/¹⁴⁴Nd.

Lead and strontium isotope ratios increase along the Reykjanes Ridge towards Iceland^{9,21}, suggesting a coupling of the thermal anomaly (that is, crust thickness and by isostasy, ridge depth) with the geochemical anomaly (the enriched signature) which has been explained as a mixing of plume and MORB endmembers. The new Pb isotope data for the high-MgO lavas are compared with existing data for recent Icelandic basalts and Atlantic MORB in Fig. 4c. The lavas of the high-MgO suites not only have depleted Pb isotope ratios with respect to the Icelandic basalt field, but overlap the fields for their adjacent ocean ridges. Moreover the lowest-FeO (highest-MgO) lavas from Theistareykir and the Reykjanes Peninsula have lead isotope ratios as depleted as the most depleted MORB endmembers from the Kolbeinsey Ridge (D. F. Mertz and C. W. Devey, personal communication) and the Reykjanes Ridge²¹ respectively.

The ¹⁴³Nd/¹⁴⁴Nd ratios of the high-MgO suites are also depleted with respect to typical Icelandic basalts, although the most depleted values (for the lowest-FeO lavas) are not as high as the MORB endmembers of the adjacent ridges (Fig. 4b). The variation of ⁸⁷Sr/⁸⁶Sr correlates inversely with ¹⁴³Nd/¹⁴⁴Nd (not shown, see Table 1), as is consistent with other recent data²², and as with the Nd isotope ratios, the most depleted ⁸⁷Sr/⁸⁶Sr of the high-MgO suite (0.7029) is not as low as has been observed in MORB endmembers of the Kolbeinsey and Reykjanes Ridges (0.7028, D. F. Mertz and C. W. Devey, personal communication; and 0.7026, ref. 9). Nevertheless, as with the incompatible element ratios, all three isotopic tracers highlight a decoupling of the geochemical signatures of plume melts inferred from major and incompatible elements respectively.

Dynamic melting processes

The geochemical variations observed in the Icelandic high-MgO suites cannot be explained by fractional crystallization or variable degrees of batch melting. The variations in FeO content of the high-MgO lavas suggest a polybaric melting process, because at constant pressure the FeO content of a basaltic melt from a mantle source is little affected by the degree of melting^{4,5,7}, but at higher pressures, partial melts have higher FeO contents than at lower pressure^{4,5,7}. The FeO variations shown by the high-MgO lavas could thus be the result of mixing FeO-rich melts from depth with shallow FeO-poor melts. Dynamic melting models invoke melting over a range of depths (pressures), and

have been used successfully, for both geophysical and geochemical reasons, to explain MORB genesis^{2,3,23,24}.

Dynamic melting is more fully described elsewhere^{1,3,24}, but a sketch model is shown in Fig. 5. Adiabatically upwelling mantle intersects its solidus, and melts along its flow-line for the rest of its ascent. Estimated rates of melt extraction in silicates suggest that the melt produced will be extracted continuously during ascent and that the porosity of the melting column²⁵ will be small (<2%). It is envisaged that all 'instantaneous' melts are continuously extracted from all levels, mixed and erupted as an average (termed 'point and depth average' in ref. 2) of the entire column production. The system is in steady state, with unmelted mantle entering the bottom of the melting column as the mantle residue leaves the top of the column.

In such a model the deeper, FeO-rich magmas represent the first melts of 'fertile' mantle entering the bottom of the melting column, and so they will have the highest incompatible-element

contents. Conversely, the shallow, FeO-poor magmas from the top of the melting column are derived from mantle that has already undergone melt extraction throughout the underlying melting column, and so the FeO-poor magmas have very depleted incompatible-element contents. Such depletion will be greatest for the most incompatible elements. This model readily explains the variations in incompatible elements in the high-MgO suite shown in Figs 2 and 3. Notably, all the lavas of the high-MgO suites are depleted with respect to an enriched MORB (E-MORB) composition¹⁶ (Fig. 3a) that is typical of the more abundant, lower-MgO Icelandic tholeiites²⁶. This suggests that none of the high-MgO lavas sample the most enriched instantaneous melts from the bottom of the melting column, as illustrated in Fig. 5.

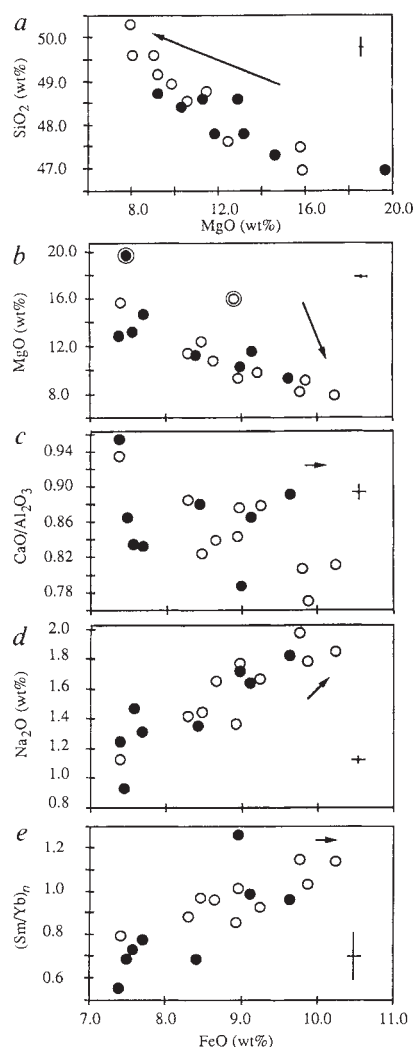


FIG. 2 Major and trace element variation. *a*, SiO₂ content against MgO. *b*–*e*, MgO, CaO/Al₂O₃, Na₂O and Sm/Yb, respectively, against FeO content. Circled symbols in (*b*) are petrographically identified as olivine cumulates. Sm/Yb in (*e*) is normalized to chondritic abundances¹⁶. Data from the Reykjanes Peninsula samples are plotted as closed symbols, data from the Theistareykir samples as open symbols. Analytical techniques are summarized in Table 1, except for Sm and Yb which were measured by instrumental neutron activation analysis⁴⁰. Analytical uncertainties assessed from 2σ deviation of triplicate sample runs are shown by crosses. Vectors show the effects of 15% fractional olivine crystallization for which olivine compositions are calculated to be in equilibrium with the evolving liquid at the start of each 0.1% increment.

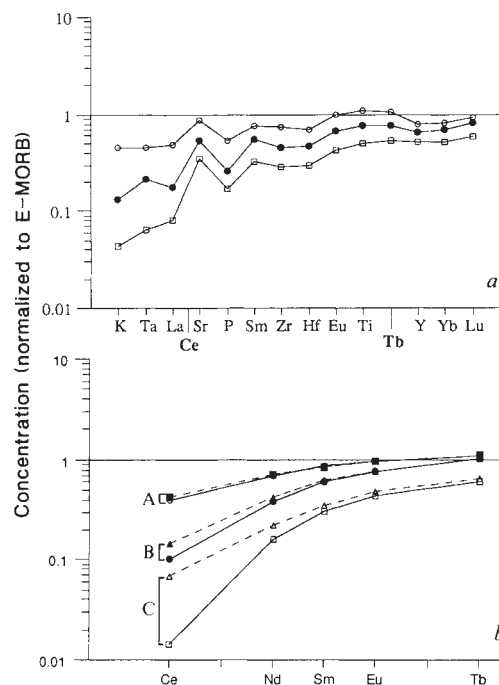


FIG. 3 Incompatible-element concentrations normalized to E-MORB¹⁶. Elements are plotted from left to right in decreasing order of inferred incompatibility during mantle melting¹⁶. *a*, Three lavas from Theistareykir which cover the range of FeO in the high-MgO suites. ○, IT2 (9.8% FeO); ●, TH8 (8.5% FeO); □, TH29 (7.4% FeO). (Several elements frequently used in such plots are excluded because of analytical difficulties in their measurement at low concentration in the high-MgO suites). Potassium, phosphorus and titanium were measured by XRF on fused beads; strontium, zirconium and yttrium by XRF on pressed powder pellets; and the rare earth elements tantalum and hafnium by instrumental neutron activation analysis⁴⁰. *b*, Three 'synthetic' plume melts (solid lines) and these melts mixed with 10% N-MORB (dashed lines). Ratios of ¹⁴³Nd/¹⁴⁴Nd and ²⁰⁶Pb/²⁰⁴Pb for resulting synthetic melt/MORB mixtures are: A, 18.60 and 0.51301; B, 18.45 and 0.51305; C, 18.27 and 0.51311. The synthetic melts are calculated from melting a mantle source with element concentrations initially that of the primitive mantle¹⁶, in 0.04% fractional melting increments (leaving a residual 0.05% porosity) for a total of 17% melting. Fractional melts are calculated from equations of Shaw⁴¹. Initially mineral proportions (*F*) are *F*_{cpx} = 0.15, *F*_{gt} = 0.1, *F*_{others} = 0.75 and melting modes (*P*) are *P*_{cpx} = 0.4, *P*_{gt} = 0.4, *P*_{others} = 0.2. After 5% melting, the upwelling mantle is no longer in the garnet stability field⁴², and ol + gt = 2.50px + sp + 0.75cpx (ref. 42). Subsequently *P*_{cpx} = 0.65, *P*_{others} = 0.35. (Distribution coefficients taken from ref. 42). The synthetic melts plotted represent summed compositions of the 0.04% increments added from the top, down to three different depths of the melting column (after the source has undergone 2, 5 and 11% melting), illustrated on Fig. 5. These melts are mixed with N-MORB melts¹⁶. The isotope ratios of the mixtures are calculated assuming lead has the same incompatibility as cerium during mantle melting³⁰ and the plume has ¹⁴³Nd/¹⁴⁴Nd = 0.51295, ²⁰⁶Pb/²⁰⁴Pb = 18.7 whereas MORB has ¹⁴³Nd/¹⁴⁴Nd = 0.51325, ²⁰⁶Pb/²⁰⁴Pb = 18.1 (the lead isotope values are therefore appropriate for the Reykjanes Peninsula suite).

Indices of major element depletion are also related to the FeO content of the lava. Figure 2c shows that the lowest-FeO lavas have high $\text{CaO}/\text{Al}_2\text{O}_3$ (>0.85), similar to melts produced from the depleted Tinaquillo lherzolite⁷, whereas the higher-FeO lavas have more typical MORB values. The ratio $\text{CaO}/\text{Al}_2\text{O}_3$ is generally higher in the instantaneous melts generated at shallower levels in the melting column, because CaO is buffered by the presence of residual clinopyroxene throughout much of the melting column, whereas Al_2O_3 is largely incompatible once any aluminous mantle phase is exhausted, which occurs after smaller degrees of melting, and hence nearer the bottom of the melting column.

This dynamic melting model accounts for the large, correlated variations of FeO with incompatible-element concentrations and ratios, and it is similar to a model proposed by Wood²⁷. It cannot readily account, however, for the strong correlation between FeO and MgO. Experimental batch-melting studies, summarized

by Klein and Langmuir³, show that the MgO content of instantaneous melts varies little with depth in the melting column. Instead the MgO contents of the erupted lavas, from mantle of a given temperature, will primarily reflect the amount of olivine fractionation experienced. It is therefore surprising that in these Icelandic suites, MgO content, which is controlled by olivine fractionation, correlates so well with FeO content, which is a function of the mean depth of partial melting of the magma.

This can be reconciled by examining the shallow-level mixing processes which determine the proportions of instantaneous melts from different depths that constitute any erupted magma. Higher-density magmas will collect at the base of a mixing zone or magma chamber²⁸, and as the higher-FeO melts from the bottom of the melting column will be denser than those with lower FeO (at similar MgO) produced from the top of the melting column, they will not mix readily. Sparks *et al.*²⁸ argued that picritic melts would be trapped at the base of a more evolved, basaltic mid-ocean magma chamber until they had fractionated sufficient olivine to reduce their density enough to mix with the other magmas. The situation envisaged for the Icelandic high-MgO suites is similar, but with picrites richer in FeO being trapped beneath picrites less rich in FeO. The deepest, highest-FeO instantaneous melts are the densest melts and must therefore fractionate the most olivine to mix and erupt. As the highest-FeO lavas must have the greatest proportion of such deep dense melts, these magma mixtures must have undergone the greatest net amount of olivine fractionation before eruption and hence have the lowest MgO contents. In contrast, the lowest-FeO (highest-MgO) magmas, which have experienced little fractionation, represent almost primitive instantaneous melts from the top of the melting column.

Plume-MORB interaction

Although the correlations between major elements and incompatible-element contents and ratios the high-MgO suites can be modelled by melting and mixing processes of melts from a homogeneous source, the isotope variations require contributions from isotopically distinct sources. The depleted isotope signature of the high-MgO lavas with respect to the typical Icelandic tholeiites suggest the involvement of an N-MORB source, and recent models show entrainment of MORB-source mantle into a rising plume²⁹, which will subsequently melt as it wells up beneath a ridge axis.

For a given degree of MORB contamination, the lavas most depleted in incompatible elements will show the greatest isotopic change. Additionally, such contamination will have a greater effect on Pb than on Nd isotope ratios, because the former element is more incompatible in the upper mantle, and therefore more depleted in the high-MgO lavas. This explains why the Pb isotope ratios in the lowest-FeO basalts of the Theistareykir and Reykjanes Peninsula suites are as unradiogenic as the MORB endmembers of the Kolbeinsey Ridge and Reykjanes Ridge respectively, whereas the corresponding highest $^{143}\text{Nd}/^{144}\text{Nd}$ are not as depleted as Nd in the adjacent MORB.

Figure 3b shows some forward-modelled rare earth element concentrations, normalized to E-MORB, as in Fig. 3a. The three synthetic lava compositions shown are calculated by adding all instantaneous melts from the top down to three different depths within the melting column (considered only in one dimension for simplicity), as illustrated in Fig. 5. The effect of adding 10% N-MORB melt¹⁶ to these synthetic plume melts is also shown in Fig. 3b, together with the isotope ratios of the mixtures (assuming lead has incompatibility equal to that of cerium during mantle melting³⁰ and using the plume and MORB isotope ratios given in the figure caption). Notably, adding 10% MORB raises the concentrations of highly incompatible elements in the very depleted synthetic melts to values similar to those observed in the high-MgO lavas. Hence in the very depleted lavas the Ce concentrations and, by inference, the Pb isotope ratios are dominated by the MORB signature, whereas Nd contents and

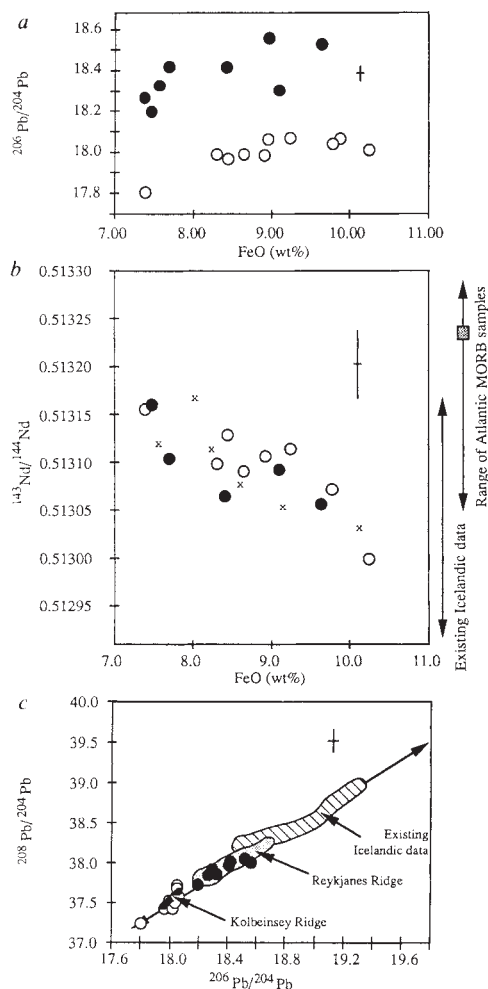


FIG. 4 Concentration of FeO against a, $^{206}\text{Pb}/^{204}\text{Pb}$ and b, $^{143}\text{Nd}/^{144}\text{Nd}$. Range of Atlantic MORB $^{143}\text{Nd}/^{144}\text{Nd}$ data from refs 12 and 44–46. Crosses show data for other similar Reykjanes Peninsula lavas^{17,19}. The shaded box indicates $^{143}\text{Nd}/^{144}\text{Nd}$ for MORB endmembers for Reykjanes Ridge and Kolbeinsey Ridge (ref. 44 and D. F. Mertz and C. W. Devey, personal communication, respectively). c, Ratio of $^{206}\text{Pb}/^{204}\text{Pb}$ against $^{208}\text{Pb}/^{204}\text{Pb}$. The hatched region shows existing data for post-glacial Icelandic basalts¹¹, the shaded region shows data for the submarine Reykjanes Ridge basalts²¹, and the bar shows the range of lead isotope values for samples from the Kolbeinsey Ridge (D. F. Mertz and C. W. Devey, personal communication). The double-headed arrow shows the range of lead isotope values^{21,44,45,47–49} for Atlantic MORBs and is aligned to give the best fit to the data. Symbols for Reykjanes Peninsula and Theistareykir suites are as in Fig. 2. Analytical techniques are described in Table 1. Analytical uncertainties (2σ deviation of replicate analyses of standards) are shown by crosses.

isotope ratios are less affected. A more comprehensive treatment of these mixing processes is in preparation.

As MORB from the Kolbeinsey and Reykjanes Ridges have distinct Pb, but similar Nd isotopic compositions, contamination of the high-MgO lavas by their respective adjacent MORB reservoirs results in distinct Pb isotope fields for the Theistareykir and Reykjanes suites but just one Nd array. What is less clear is why the Pb isotope ratios of the two suites do not converge towards a single plume isotopic composition at high FeO, but diverge towards apparently distinct endmembers (Fig. 4a), suggesting Pb isotope heterogeneity in the plume on a scale of several hundred kilometres. Although they analysed samples of various ages, Welke *et al.*³¹ also observed that the Pb isotope ratios in samples from the south and west of Iceland were more radiogenic than those from the north and east.

Although mixing between plume and small amounts of MORB melts is required to explain the isotope variation observed in the high-MgO suite, it is important to stress that the principal control on the geochemical variation is nevertheless the melting processes described in the previous section, and not simple mixing between MORB and a plume rich in FeO and incompatible elements. For example, in such a mixing model, the Pb isotope ratios of the lowest-FeO lavas of the suites would suggest that they represented pure MORB endmember compositions, but the $^{143}\text{Nd}/^{144}\text{Nd}$ (and $^{87}\text{Sr}/^{86}\text{Sr}$) data of the same samples is incompatible with this (Fig. 4).

Implications of the melting model

The models of McKenzie and Bickle² and more recent refinements of Watson and McKenzie³² suggest that the high-degree melts required to generate the thickness of the Icelandic crust must have a composition with more than 15% MgO. Their calculations estimate the sum of all the instantaneous melts from the whole melting column, and therefore a high-MgO melt calculated in this way is also expected to possess enriched incompatible-element ratios, characteristic of the enriched plume. In Meyer's comprehensive data set of Icelandic rift basalts²⁶, although most of the basalts have enriched, E-MORB like incompatible-element ratios, none of these enriched lavas has more than 9% MgO. Conversely, the high-MgO basalts of this study all have depleted incompatible-element ratios with respect to E-MORB (Fig. 3a).

We have argued above that the FeO, MgO and SiO₂ variations in the high-MgO suite suggest that the dense, FeO and incompatible-element-rich melts from the bottom of the melting column must fractionate olivine if they are to be sampled at the surface (as mixtures with shallower melts from the column). Thus, although a theoretical 'parental' melt from the Iceland plume is both enriched in incompatible-element ratios and has high MgO content (>15%), this is never realised in practice because to mix all of the independently segregated instantaneous melts to produce a point-and-depth average, some olivine fractionation must occur. Thus the more common, fractionated (<9% MgO) Icelandic basalts sample instantaneous melts from the whole of the melting column, and so erupt with a full complement of incompatible elements from the melting column (and hence enriched incompatible-element ratios), but without the full budget of olivine-compatible elements. More rarely, as observed in this study, melts from the upper portion of the melting column can erupt as lavas with more primitive MgO contents, by escaping mixing (and accompanying fractionation) with the complementary enriched melts from the bottom of the melting column, but they carry only a small proportion of the incompatible elements expected for a point-and-depth average of the whole melting column.

Hence although the high-MgO lavas have primitive Mg#, their compositions cannot be inverted to deduce information about the Iceland plume source. But if, as believed, the MgO contents of instantaneous melts remain constant throughout the melting column, the least-fractionated lavas of this study provide

an important constraint on the MgO content of plume-derived melts. It might be argued that the composition of the lavas with the highest MgO contents of the Theistareykir and Reykjanes Peninsula suites do not represent liquid compositions, but indicate cumulus olivine (as for the Réunion picrites³³). But although two samples clearly are olivine cumulates (Fig. 2b), the other samples all lie on coherent geochemical trends that cannot be explained by olivine addition. Moreover, the forsterite contents of the olivine phenocrysts of the most MgO-rich lavas imply equilibrium with liquids of 12–14% MgO. It is therefore reasonable to argue that the high-MgO endmember of these trends represents a liquid composition with 14–16% MgO. This is similar to estimates of MgO content for Hawaiian plume melts^{31,34,35} and hence suggests a similar temperature for both plumes.

Although melts from beneath Iceland are derived from hotter and geochemically enriched mantle, geophysically similar processes occur beneath all spreading ridges. Noticeably, the correlation between FeO and Na₂O in the high-MgO suites is consistent with the vector of local variation observed in individual ridge segments from a global MORB database³⁶.

In some MORB suites, trends of decreasing CaO/Al₂O₃ with increasing TiO₂ contents and decreasing Mg# have been explained by clinopyroxene fractionation, even though no clinopyroxene phenocrysts are observed, or even predicted for such melt compositions^{37,38}. But for the Icelandic high-MgO suites it is argued that CaO/Al₂O₃ variation is a partial melting effect in which decreasing CaO/Al₂O₃ correlates with increasing TiO₂, thus mimicking a fractionation trend involving clinopyroxene. Furthermore, the lavas with lower CaO/Al₂O₃ also represent a melt mixture with a larger proportion of melts from the bottom of the melting column, which had to fractionate more olivine before mixing could occur. Thus, the partial melting effects controlling CaO, Al₂O₃ and TiO₂ are accompanied by a decrease in MgO resulting from the removal of olivine. This combination generates the chemical features that might be attributed to clinopyroxene fractionation without the involvement of this phase.

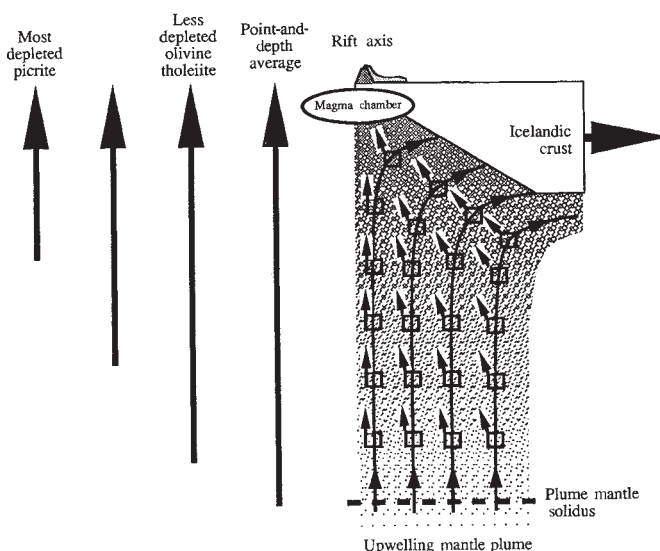


FIG. 5 Sketch of idealized dynamic melting column. Melting and melt segregation occur continuously throughout ascent of mantle along the streamlines (shown as solid lines), but are illustrated only at specific points (shown as boxes), with arrows indicating direction of focused segregation of the 'instantaneous' melts. Increasingly darker shading represents increasingly melt-depleted mantle. The heavy arrows to the left of the melting column show the depth to which instantaneous melts are sampled for the range of lava compositions represented in the high-MgO suites (and calculated in Fig. 3) and for the point-and-depth average².

The Icelandic high-MgO suites display large variations in incompatible-element contents and ratios as a result of mixing various proportions of instantaneous melts from different depths of the melting column. We argue that most MORBs may represent a similar 'cocktail' of melts segregated from different depths and mixed in an axial chamber. Although a local average

of numerous MORB compositions will reflect the incompatible element ratios of the point-and-depth-averaged melt of the underlying mantle, chemical variability of individual lavas is likely to result from different mixes of melts, segregated from various levels, feeding the magma chamber at any given instant. □

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Two different microtubule-based motor activities with opposite polarities in kinetochores

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The movement of microtubules on the kinetochores of isolated chromosomes has been examined by video microscopy. Two different microtubule-based motors on the kinetochore were identified, which have opposite directions of movement. The activities of these two motors can be regulated by factors that can influence phosphorylation.

CHROMOSOMES segregate at mitosis by interacting with the microtubules of the mitotic spindle. The interactions that result in correct segregation occur mainly at the kinetochore, a specialized region of the chromosome that overlies the genetically defined centromere^{1,2}. Mitotic spindle microtubules have a defined polarity: their minus-ends are attached to the centrosomes at the spindle poles, whereas a subset of the plus-ends interact with the kinetochores³. After microtubule attachment the kinetochores move in complex patterns, resulting in the segregation of the chromosomes. At prometaphase they move both towards and away from the closest spindle pole until the oscillatory movements of the chromosomes are centred on the metaphase plate, a plane perpendicular to the long axis of the spindle. Anaphase begins with the splitting of the sister

chromatids, and the chromosomes then progress steadily towards the poles⁴.

During chromosome movements, kinetochore microtubules change their length by adding or losing subunits at the ends attached to the kinetochores. At the same time, the kinetochores slide over the microtubule themselves^{5–7}. This combination of polymerization and depolymerization, coupled with sliding at kinetochores, has led to two different models to explain the mechanism of chromosome movement. One model proposes that the energy released during microtubule polymerization and depolymerization at the kinetochore (which comes from GTP hydrolysis) could drive chromosome movement directly^{5,7–9}.

The other model proposes that movement is driven by motor proteins¹⁰ which generate force to move with a defined polarity on the microtubule, by the hydrolysis of ATP^{11–14}. The role of motors is strongly implicated by two observations. First, when kinetochores first attach to microtubules they make a lateral connection, followed by polewards sliding of the kinetochores, which suggests a minus-end motor activity¹¹. Second, dynein, a minus-end-directed motor, is a component of the prometaphase kinetochore^{12,13}, and a functional plus-end-directed motor has been identified on the kinetochores of isolated chromosomes¹⁴.

It is likely that both microtubule dynamics and motor activities will play important parts in chromosome movement. Under-

standing the mechanism of chromosome movement and its regulation will require the dissection of the various activities of the kinetochore and then the determination of how they operate together to achieve chromosome segregation. Owing to the complexities of spindle organization *in vivo*, attempts have been made to reconstitute kinetochore-microtubule interactions *in vitro*^{7,9,14,15} (for a review, see ref. 16).

Here we extend the analysis of the role of microtubule-based motors in mitosis by examining their function on the kinetochores of isolated chromosomes. By analysing the movement of microtubules directly by using fluorescence video microscopy we demonstrate that kinetochores contain two motors, which move microtubules in an ATP-dependent manner, but which have opposite polarities. Which one of the motors is active on the kinetochores can be regulated by factors which influence phosphorylation state. We propose that the kinetochore actively controls the direction of chromosome movements using protein phosphorylation.

Establishment of the assay

We wanted to find out whether functional microtubule-based motors are a component of the kinetochore. Microtubule-based motors have defined movement polarity on the microtubule¹⁰ and therefore it was necessary to design a microtubule whose polarity could be determined directly under the microscope. Dimly labelled rhodamine tubulin was polymerized from the ends of brightly labelled rhodamine microtubule seeds (Fig. 1a, S) so that the plus-ends grew longer than the minus-ends (as indicated in Fig. 1a)¹⁷.

To attach these segmented microtubules to the kinetochores we took advantage of the fact that pre-formed microtubules will bind specifically to kinetochores *in vitro*⁹. Mammalian chromosomes, isolated from cells arrested at metaphase, were perfused into a thin chamber, where they stuck to the glass slide. Next, the bright microtubule seeds were perfused into the chamber, where they were captured by the kinetochores (step A, Fig. 2g). Uncaptured seeds were washed out and dimly labelled rhodamine tubulin was perfused in to polymerize from the seeds that had bound to the kinetochores (step B, Fig. 2g). The resulting microtubules were stabilized with a buffer containing taxol. We were then able to assay motor activity in the absence of microtubule polymerization dynamics.

The kinetochores could easily be identified because the marked microtubules attach to kinetochores at their brightly labelled seeds (Fig. 1b). The chromosome was visualized with the DNA dye Hoechst (arrow, Fig. 1c), which allowed us to confirm that the clusters of seed attachment always correspond to the expected position of the kinetochores on the chromosome, a large constriction.

Kinetochores contain a functional minus-end motor

To assay for motor activity, ATP was perfused into the chamber containing microtubules bound to the kinetochores. After the addition of ATP, microtubules moved relative to the kinetochore, demonstrating the presence of microtubule-based motors on the kinetochore.

Under these conditions, the microtubules move across the stationary kinetochore with the minus-end trailing and the plus-end leading (Fig. 2a–e, and step C in g). Therefore there must be motors attached to the kinetochore which move microtubules by minus-end-directed movement. Most microtubules commence moving along the kinetochore immediately after ATP addition (Fig. 2f) and they slide along at $28 \pm 0.4 \mu\text{m min}^{-1}$ ($n = 11$) at room temperature until their minus-end is reached; a few stick with their ends at the kinetochore, but most detach and diffuse away.

Kinetochores also contain a plus-end motor

Many events at mitosis are controlled by phosphorylation¹⁸, and we tested its role in the control of motor activity. ATP- γ -S, an

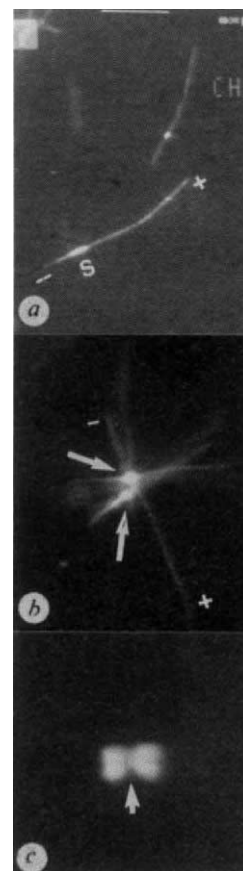


FIG. 1 Attachment of polarity marked microtubules to kinetochores. *a*, Polarity-marked microtubules. The longer plus-end (+) and the short minus-end (–) have grown from the bright seed (S)¹⁷. *b*, *c*, Marked microtubules captured by kinetochores (see step A, *g*). *b*, Marked microtubules in the rhodamine channel. The kinetochores, arrows, are visualized by the clusters of captured seeds (S, *a*). *c*, DNA, stained with Hoechst, of the same chromosome. The primary constriction (arrowhead) is the location of the kinetochores. To check the alignment of the seed clusters with the constriction, about 100 chromosomes with an obvious constriction were selected in the Hoechst channel and the attachment of the microtubules examined in the rhodamine channel. In all cases the cluster of seeds was located at the primary constriction. Furthermore, in no case did we see more than two clusters of seeds on the chromosome. About one in every two chromosomes had a microtubule bound to the chromosome arms, but these microtubules were never in clusters. About 5% of chromosomes had microtubules bound all over their surface. These were always ignored in the assay for kinetochore activity. Scale bar, 10 μm .

METHODS. Rhodamine tubulin was prepared as described¹⁷. Chinese hamster ovary chromosomes were prepared as described⁹, except that swelling of the chromosomes occurred at 30 °C, and the chromosomes were stabilized by 4 mM MgCl₂ instead of by polyamines. Chromosomes were diluted 1/300 in PME (10 mM potassium-PIPES, pH 7.4, 4 mM MgCl₂, 1 mM EGTA, 1x protease stocks (PI), 5 mM DTT; 100x PI: 10 mg ml⁻¹ leupeptin, pepstatin, chymostatin containing 10% bovine serum albumin). Perfusion chambers were constructed using two strips of double-sided sticky tape on a glass slide to support a coverslip. Diluted chromosomes were perfused in at 4 °C. All subsequent experiments describe complexes attached to the glass slide. The chamber was washed twice (one wash volume = one chamber volume) in PMD (80 mM potassium-PIPES, pH 6.8, 1 mM EGTA, 4 mM MgCl₂, 5 mM DTT) with 1x PI and shifted to 37 °C. To initiate the capture reaction (Fig. 2g), GMPCPP seeds⁴², diluted into PMD with 1x PI, were perfused in at 37 °C for 5 min. Uncaptured seeds were washed out with PMD and 1 mM GTP. A mixture of cyclized-tubulin/rhodamine-tubulin⁴² at 10:1 μM was diluted in PMD with 1 mM GTP and 0.1x PI, and grown from the ends of the kinetochore-attached GMPCPP seeds (Fig. 2g). The resulting microtubules were stabilized by washing in antifade buffer⁴³ (PMD and 0.2% 2-mercaptoethanol, 20 μM taxol, 5 $\mu\text{g ml}^{-1}$ glucose oxidase, 10 $\mu\text{g ml}^{-1}$ catalase, 10 mM glucose, 0.1x PI) with 1 $\mu\text{g ml}^{-1}$ Hoechst 33342. A detailed protocol is available on request.

ATP analogue, can be used by kinases to thio-phosphorylate their substrates¹⁹ irreversibly because thio-phosphate groups are not easily removed by protein phosphatases²⁰. ATP- γ -S was included during the capture reaction (step A, Fig. 2g), the chromosomes were then washed extensively and ATP was added to investigate the activity of the motors. We found that if the kinetochores were pre-treated with 300 μ M ATP- γ -S in the capture reaction, addition of 1 mM ATP induced all the microtubules to move by plus-end-directed rather than minus-end-directed movement (Fig. 3a-d; Fig. 2g). This plus-end-directed motor is probably identical to that identified previously in a more indirect assay⁹. We therefore conclude that the kinetochores of isolated mitotic chromosomes contain both minus- and plus-end-directed motors and that pre-treatment with ATP- γ -S turns off the minus-end-directed motor and turns on the plus-end-directed motor. The role of phosphorylation in control of motor activity is described in more detail below.

The characteristics of the plus-end and minus-end motors are different: the velocity of plus-end-directed movement is only $2.9 \mu\text{m min}^{-1} \pm 1.5$ ($n = 48$) at room temperature compared with $28 \mu\text{m min}^{-1}$ for the minus-end-directed movement; plus-end movement commences at 100 μ M ATP, whereas minus-end-directed movement requires only 1 μ M ATP.

Nucleotide requirements of the motors

To relate the kinetochore motors to already known purified motors, we tested the response of the kinetochores to a group of ATP analogues which are used differently by the dynein and kinesin ATPases²¹. The two kinetochore motors respond differently to these analogues (Table 1), implying that they are different molecular species. The spectrum of response of the minus-end motor is identical to that of dynein. Further evidence for its identity is that the chromosomes used in the assay are isolated in a state corresponding to prometaphase, and dynein is localized on the kinetochores of prometaphase chromosomes in both whole cells and isolated chromosomes by immunofluorescence^{12,13}. It seems less likely to be a homologue of claret, another minus-end-directed cytoplasmic motor, because claret moved microtubules at only $6 \mu\text{m min}^{-1}$ (ref. 22). The plus-end motor has characteristics different from those of conventional kinesin. In particular, GTP is used by kinesin, but is not used by the kinetochore plus-end-directed motors to generate movement. Kinesin also moves microtubules much faster than the kinetochore motor²³ and has not been identified as a kinetochore component by immunofluorescence or genetics^{24,25}. The plus-end motor may be encoded by a novel member of the kinesin gene family now being identified²⁶⁻²⁸, and an analysis of the

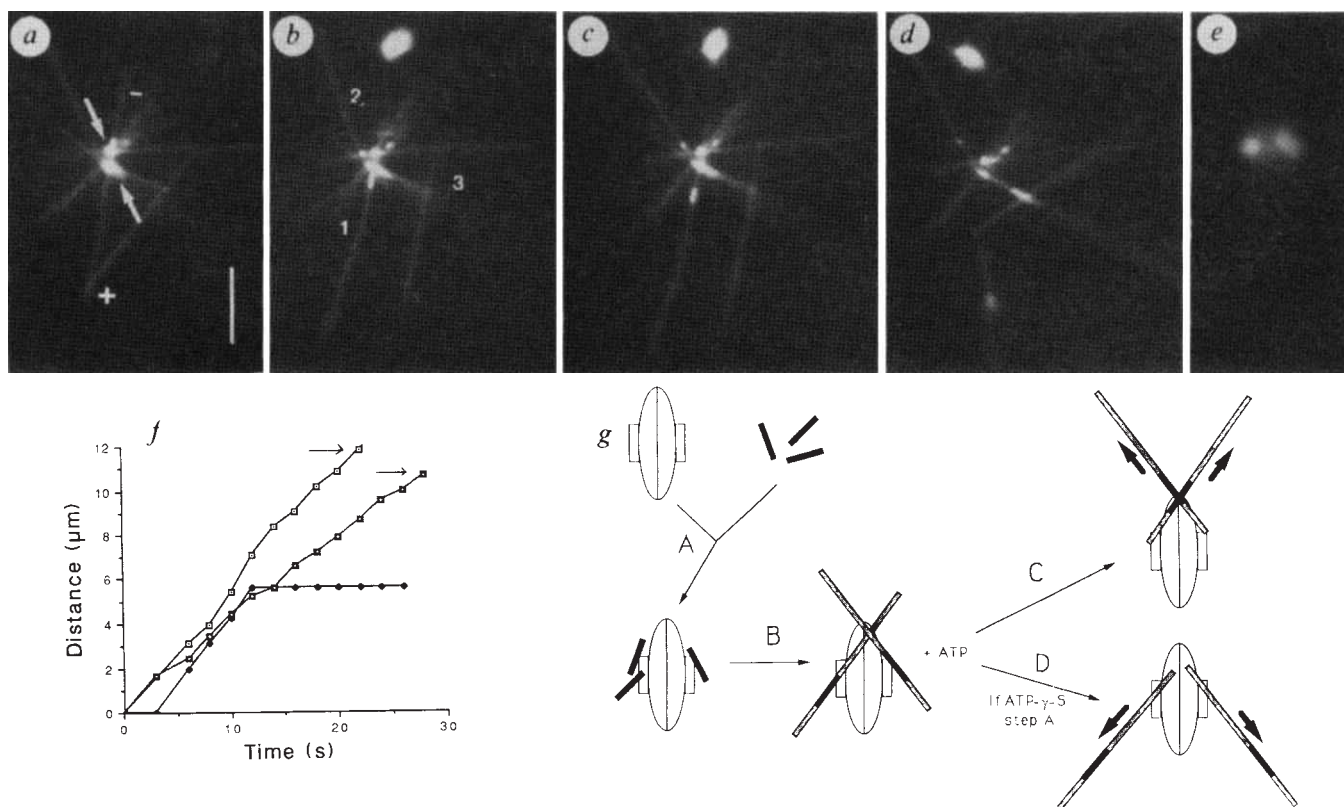


FIG. 2 Minus-end-directed movement of microtubules on isolated kinetochores. *a*, Complex after attachment of segmented microtubules but before ATP addition (see Fig. 1*b*). *b-d*, Behaviour of three microtubules (1-3) after 1 mM ATP addition. *b*, 5 seconds: microtubule 1 has commenced minus-end movement and the bright seed can be distinguished from the kinetochore, whereas the minus-end has moved closer to the kinetochore. The dimly labelled portion of the microtubule is more easily seen during movement. *c*, 10 seconds: seed of microtubule 1 has moved further from kinetochore. Microtubule 2 is undergoing minus-end movement. *d*, 30 seconds: microtubule 1 has fallen off, microtubule 3 has continued to move by minus-end movement. *e*, Hoechst image of chromosome. Arrows, kinetochores; +, plus-end of microtubule; -, minus-end of microtubule. *f*, Graph of the behaviour of three microtubules from one kinetochore (different from *a-e*) after addition of ATP. The graph was constructed by measuring the distance between the kinetochore and the brightly labelled portion of the microtubule. Arrows mark the time of microtubule detachment when

the minus-end reached the kinetochore. *g*, Summary of the movements of microtubules on isolated chromosomes. A, capture reactions; B, addition of dimly labelled tubulin which grows off the captured seeds. The resulting plus-ends are longer than the minus-ends. After addition of ATP, microtubules move by minus-end-directed movement (C) or plus-end-directed movement (D) after addition of ATP- γ -S at step A. Scale bar, 10 μ m.

METHODS. ATP was diluted to 1 mM in antifade and kept at 4 °C. A complex was selected in the rhodamine channel, by looking for the two foci of bright seeds (Fig. 1*b*). Recording was started, the diluted ATP was perfused into the chamber, and the complex quickly brought back into focus. After the recording was finished, the presence of a chromosome was confirmed in the Hoechst channel. Recording was with a SIT camera and images stored on an OMDR Panasonic TQ3038. The light dose was limited by shuttering, coordinated with a Datacube Maxvision image processor using custom-written software (available on request).

substrate specificity of these kinesin homologues may help elucidate whether this is true.

Motor activities and phosphorylation

To investigate further the effect of ATP- γ -S in regulation of motor activity, we decreased the amount of ATP- γ -S added during the capture reaction and measured the number of microtubules moving by plus- or minus-end-directed movement. We observed a reciprocal relationship between the amount of minus- and plus-end-directed movement as the ATP- γ -S concentration was varied (Fig. 4a). Although ATP- γ -S could be acting as a competitive inhibitor for ATP at the motor active site²⁹, extensive washing between capture and ATP addition showed that this is unlikely. We therefore suspected that ATP- γ -S was affecting the activity of the kinetochore motors by changing the phosphorylation state of the kinetochore.

To test further this possibility we used an inhibitor of mitotic phosphorylation, 6-dimethylaminopurine (DMAP)³⁰. The ability of DMAP to inhibit the activation of plus-end-directed movement by ATP- γ -S was investigated by including 10 μ M ATP- γ -S and DMAP in various concentrations in the capture reaction (step A, Fig. 2g). DMAP inhibits the functional activation of the plus-end motor by ATP- γ -S (Fig. 4b).

Because it is difficult to do conventional biochemistry on isolated chromosomes, we used a cytological approach to look at kinetochore phosphorylation, taking advantage of an antibody that recognizes thio-phosphoserine epitopes^{31,32}. This antibody recognizes Chinese hamster ovary kinetochores after exposure to ATP- γ -S in concentrations sufficient to activate plus-end-directed movement³³. We assayed whether DMAP would also prevent the ATP- γ -S-dependent staining of kinetochores by incubating isolated chromosomes with 10 μ M ATP- γ -S in the presence of DMAP, and then staining them with the anti-thiophosphate antibody. By measuring the intensity of

TABLE 1 Nucleotide requirements for kinetochore motors

Nucleotide	Dynein	Kinetochore minus-end	Kinesin	Kinetochore plus-end
ATP 10 μ M	+	+	+	—
ATP 1 mM	+	+	+	+
GTP 1 mM	—	—	+	—
Monomethyl ATP	+	+	+	+
Dimethyl ATP	—	—	+	—
Ethano ATP	—	—	+	—
8-Azido ATP	+	+	—	—
Dideoxy ATP	+	+	+	+
3'Deoxy ATP	+	+	+	+

Response of the two different motors to different nucleotides. Data for kinesin and dynein taken from ref. 21. Analogues are used at 1 mM. The limitations of this method are that movement is investigated at only a single nucleotide concentration. A thorough study would require determining the Michaelis constant (K_m) for each nucleotide, which is beyond the scope of this work. Furthermore, because other ATP-using enzymes seem to be involved in controlling motor activity, they may also be affected by the analogues and therefore the response to the analogues may not be due to direct interaction with the active site. This method, however, currently represents the only fairly specific way to determine ATPase identity of novel motor proteins.

fluorescence as a function of DMAP concentration (Fig. 4b–f), we found that DMAP inhibits the thiophosphorylation of epitopes on the kinetochore. We conclude that DMAP prevents the functional activation of the plus-end motor by ATP- γ -S by inhibiting the thiophosphorylation of epitopes on the kinetochore, and therefore that phosphorylation is involved in the control of motor activity. Phosphorylation could cause the release of the minus-end motor from the kinetochore, but anti-dynein antibodies recognize the kinetochore even after ATP- γ -S treatment³³.

Control is specific to kinetochores

We tested whether ATP- γ -S affected non-kinetochore motors. Our chromosome preparations are contaminated with vinblastine paracrystals³⁴. We noticed that they also bound microtubules during the capture reaction and that subsequently the microtubules moved over them by minus-end-directed movement after addition of ATP. The velocity ($30 \pm 1.2 \mu\text{m min}^{-1}$; $n=9$) and ATP concentration required to initiate movement

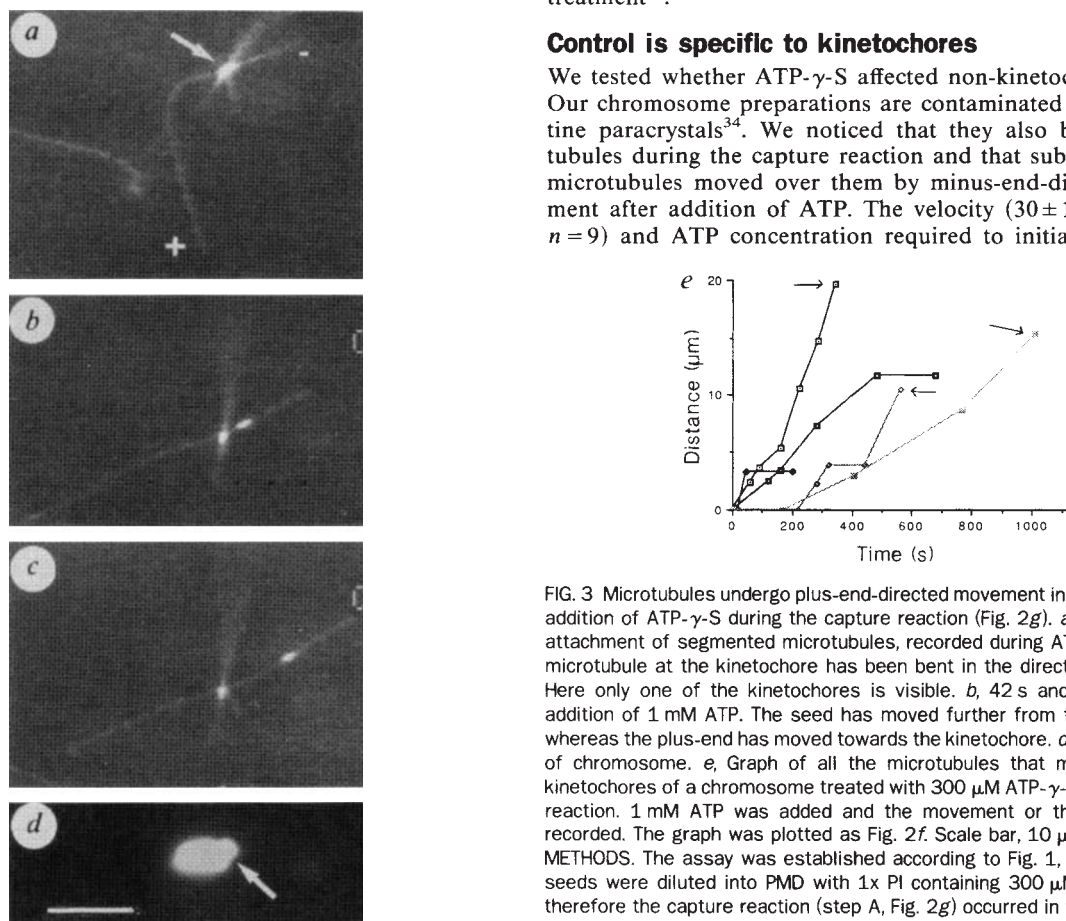


FIG. 3 Microtubules undergo plus-end-directed movement in 1 mM ATP after addition of ATP- γ -S during the capture reaction (Fig. 2g). a, Complex after attachment of segmented microtubules, recorded during ATP addition. The microtubule at the kinetochore has been bent in the direction of the flow. Here only one of the kinetochores is visible. b, 42 s and c, 110 s after addition of 1 mM ATP. The seed has moved further from the kinetochore, whereas the plus-end has moved towards the kinetochore. d, Hoechst image of chromosome. e, Graph of all the microtubules that moved from the kinetochores of a chromosome treated with 300 μ M ATP- γ -S in the capture reaction. 1 mM ATP was added and the movement or the microtubules recorded. The graph was plotted as Fig. 2f. Scale bar, 10 μ M. METHODS. The assay was established according to Fig. 1, except that the seeds were diluted into PMD with 1x PI containing 300 μ M ATP- γ -S, and therefore the capture reaction (step A, Fig. 2g) occurred in the presence of ATP- γ -S. Movement was recorded and analysed according to Fig. 2.

were similar to those of the minus-end motor on the kinetochore. Because these motors had been treated in the same way during isolation as the kinetochore motors, we examined whether the minus-end-directed motors on the paracrystals were also inhibited by ATP- γ -S. Even when kinetochore minus-end-directed movement was fully inactivated by ATP- γ -S pre-treatment, minus-end movement for the other motors was unaffected in both velocity and frequency (Fig. 5). This result confirms that ATP- γ -S causes inactivation of the minus-end motor on the kinetochore by a mechanism distinct from competitive inhibition at the active site. It also suggests that the mechanism by which ATP- γ -S acts to inhibit the minus-end movement is specific to the kinetochore. It remains unknown whether this specificity arises because the minus-end-directed motor attached to kinetochores is a different molecular species or because the regulatory kinase is specific to the kinetochore.

Discussion

We have established an assay that allows microtubule movement by kinetochores to be observed under the microscope. Using this assay we have obtained evidence for two oppositely directed

motor activities which are differentially regulated by a phosphorylation system localized to kinetochores. Owing to the complexity of mitosis *in vivo*, it has been difficult to identify the role of motors in chromosome movement. After the attachment of kinetochores to microtubules *in vivo*, at prometaphase a minus-end-directed motor is probably responsible for moving chromosomes rapidly ($20\text{--}80\ \mu\text{m min}^{-1}$) polewards¹¹. At this stage kinetochores make lateral attachments to microtubules, and the similar lateral attachment is seen during movement in our *in vitro* assay. Therefore, the prometaphase movement *in vivo* may correspond to the minus-end-directed motor *in vitro*. The role of dynein during polewards chromosome movement later in mitosis, when microtubules form both end-on and lateral connections to kinetochores, is less clear because the rates of chromosome movement during metaphase and anaphase ($1\text{--}3\ \mu\text{m min}^{-1}$) are much slower than would be expected for dynein. But it is still possible that dynein provides the force for polewards chromosome movement, with the rate of movement being regulated by other factors such as the rate of microtubule depolymerization at the kinetochore³⁵. It is more difficult to identify the function of a potential plus-end-directed motor *in vivo*. Chromosomes move actively away from poles during the establishment of metaphase, and the rate at which they do so ($1\text{--}5\ \mu\text{m min}^{-1}$) is similar to that of the plus-end motor we have identified *in vitro*, but other factors may also drive the movement away from poles *in vivo*³⁶.

Most importantly we conclude that the direction of motor movement on the kinetochore seems to be controlled by phosphorylation. We do not yet know how phosphorylation controls the motors, but there is a similar control system in melanophores,

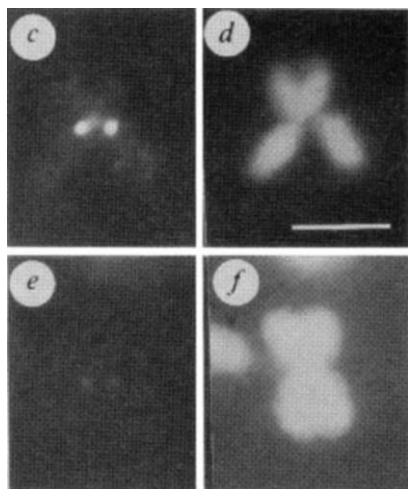
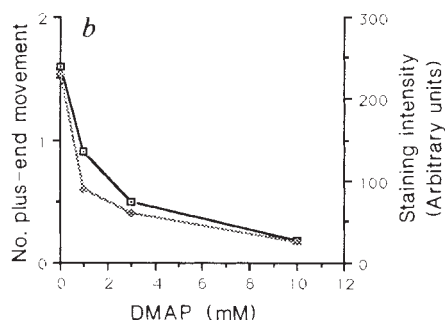
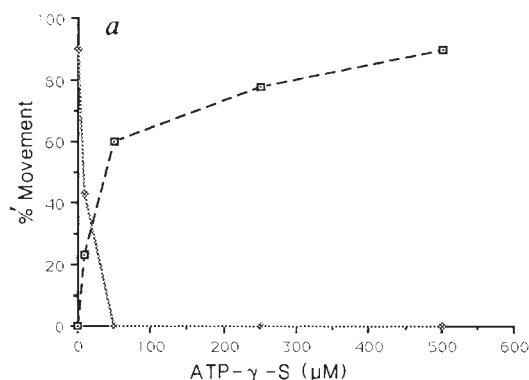
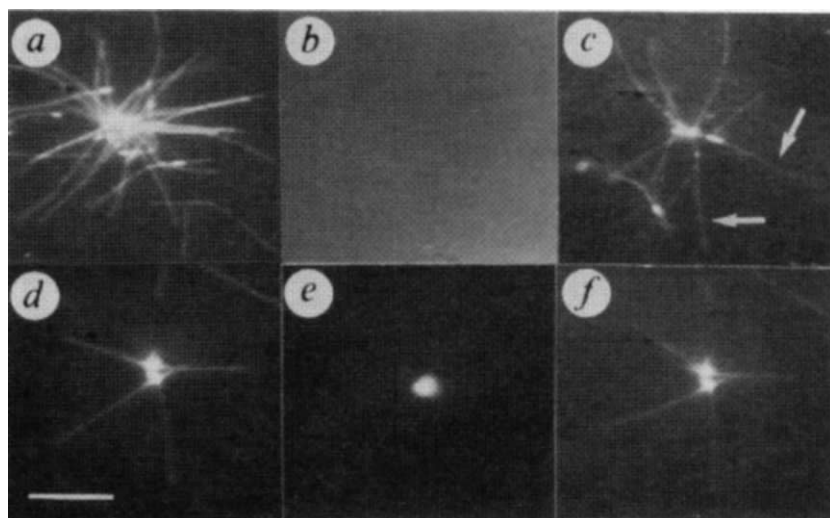


FIG. 4 Direction of movement is changed by ATP- γ -S treatment. Varying amounts of ATP- γ -S were included in the capture reaction, and the percentage of plus-end (□)- and minus-end (◇)-directed movement ascertained for each concentration of ATP- γ -S, after addition of 1 mM ATP. The number of captured microtubules was obtained by determining an average for 50 kinetochores before ATP addition. Direct observation demonstrated that all the microtubules that move by minus-end movement do so within 1 min after ATP addition (see Fig. 2). Therefore, the number of microtubules that remained captured after 1 min was counted, and the difference between this and the pre-ATP addition number taken to be the number that moved by minus-end-directed movement. After 10 min, the number moving by plus-end movement was counted. This could be counted for 50 chromosomes directly, because plus-end movement is much slower, and the plus-end of the polarity marked microtubules much longer. The actual dose-response curve varied by about 30% between chromosome preparations, but the shape of the curves and relative effect on minus- and plus-end-directed movement were always the same. *b-f*, Kinase inhibitors prevent both the activation of the plus-end motor by ATP- γ -S and the thiophosphorylation of kinetochore components. *b*, Inhibition of functional activation (■) and inhibition of thiophosphorylation of the kinetochore (◇). In this experiment to inhibit functional activation, there was 35% plus-end-directed movement at 10 μM ATP- γ -S in the absence of DMAP. *c, d*, Anti-thiophosphate (*c*) and Hoechst (*d*) staining in the absence of DMAP. *e, f*, Anti-thiophosphate (*e*) and Hoechst (*f*) stain after addition of 10 mM DMAP in the capture reaction. Scale bar, 10 μm .

METHODS. Inhibition of functional activation: plus-end-directed movement was stimulated by 10 μM ATP- γ -S in various concentrations of DMAP, and the percentage of plus-end-directed movement assayed at the different DMAP concentrations. The assay was established exactly as for Fig. 3, except that varying DMAP concentrations were added to the capture reaction along with the ATP- γ -S. Inhibition of thiophosphorylation. The assay was prepared as Fig. 3, with 10 μM ATP- γ -S in the capture reaction, and the DMAP concentration in the capture reaction was varied. They were then fixed and stained as described in ref. 33. The intensities were measured with a SIT camera and the values were therefore non-linear at higher staining intensities. To measure the average intensity, the intensity of the five highest pixels, of a 3×3 pixel area centred around the kinetochore, was averaged for 30–50 chromosomes. The intensity of staining with the anti-thiophosphoprotein antibody was then measured as a function of DMAP concentration and plotted on the graph (arbitrary units corresponding to the grey scale range of the camera, with a range of 0 to 255).

FIG. 5 Regulation by ATP- γ -S is specific to kinetochores. The assay was established according to Fig. 3a with the addition of 100 μ M ATP- γ -S during the capture reaction. The movement was then analysed after addition of ATP as in Fig. 2b. *a-c*, Movement by minus-end-directed movement on cytoplasmic remnants. Most of these remnants are probably vinblastine paracrystals³⁴, which bind dynein¹³. *d-f*, Chromosome from the same chamber. *a*, Cluster of microtubules on the paracrystal before ATP addition. The crystals were identified as a somewhat rectangular cluster of seeds. We have not formally demonstrated that they are actually paracrystals, but they were obviously different from the kinetochores, because they often bound over 40 microtubules, had no staining in the Hoechst channel, and were always of a size similar to that of paracrystals. The number of microtubules in the capture reaction was reduced to obtain this figure, because a large number of seeds on the paracrystals causes saturation of the camera. *b*, Hoechst channel. No chromosome is present. *c*, 20 s after addition of 1 mM ATP. Most of the microtubules have moved off the cytoplasmic remnant by minus-end-directed movement, and have moved out of focus. Two microtubules (marked by arrows) are undergoing minus-end-directed movement. *d*, Chromosome from the same coverslip before addition of ATP. *e*, Addition



of 1 mM ATP. The chromosome can be seen stained by Hoechst. *f*, 1 min: no microtubules have moved by minus-end-directed movement. Scale bar, 10 μ M.

where pigment movement is effected by two motors whose relative activities are controlled by phosphorylation³⁷.

The factors that determine the direction of chromosome movement *in vivo* are currently obscure, although it is clear that control of direction is essential for correct segregation. During prometaphase and metaphase, individual chromosomes behave autonomously, oscillating towards and away from poles, and eventually aligning at the metaphase plate⁴. Many models have been proposed to explain metaphase alignment³⁸⁻⁴⁰, some with passive, others with active, roles for the kinetochores in determining chromosome position on the mitotic spindle. The demonstration here that the direction of microtubule-based movement at the kinetochore can be controlled by phosphorylation implies that the kinetochore could actively be involved in determining chromosome position. By regulating the activity of the two motors according to a gradient of information from the poles, the phosphorylation system could direct the position of the chromosomes at mitosis. In our study we manipulated the phosphorylation system artificially using an irreversible kinase substrate, ATP- γ -S, but *in vivo* the level of phosphorylation is presumably regulated by a kinase-phosphatase balance¹⁸. To understand the role of phosphorylation-mediated control of

motors in positioning chromosomes, we need to know what kind of information influences this balance *in vivo*. If the kinase-phosphatase system is involved in positioning chromosomes at the metaphase plate, then it must sense the position of the chromosome in the spindle and regulate the activity of the motors accordingly. The number or tension of microtubules could provide such information. During anaphase there is irreversible polewards movement. If the kinase system plays a part in ensuring this by activating only the minus-end-directed motor, it must detect the cell-cycle stage, perhaps through the decrease in cdc2 kinase activity at anaphase⁴¹.

Such models are preliminary because the regulation of motors is more complex, involving changes in microtubule length at the kinetochore. Microtubules grow and shrink at the kinetochore during and subsequent to prometaphase in an apparently regulated fashion⁵, suggesting that motor activity and microtubule dynamics are coordinated. We examined the function and regulation of motors in the absence of polymerization dynamics. How these dynamics are regulated in conjunction with motor activities remains to be elucidated. The assay that we have developed should be an important starting point. □

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Evidence from stellar abundances for a large age difference between two globular clusters

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THE globular clusters NGC288 and NGC362 are central to recent claims¹⁻⁴ of large age differences (~3 Gyr) between globular clusters associated with our Galaxy. According to standard models for the formation of the Galaxy⁵, the system of globular clusters formed during the dynamical collapse of the protogalactic cloud, a process which should have lasted no more than 1 Gyr⁶. But the claimed age differences are derived from stellar evolution models using assumed CNO abundances, and uncertainty in the actual CNO abundances of about a factor of three could account for an apparent 2-Gyr age difference^{7,8}. We have accurately measured abundances in red giants in NGC288 and NGC362, and find that the Fe abundance and the sum of the C, N and O abundances are essentially the same in every star studied. By eliminating compositional differences and thus confirming the reality of the age difference, these results imply a cluster formation period that is hard to reconcile with the standard collapse model^{5,6}.

Much attention has been given in recent years to the accurate determination of the age of globular clusters. Absolute ages set important limits to fundamental cosmological parameters, for instance, a lower limit to the age of the universe and hence an upper limit to the Hubble constant, whereas the spread in age among galactic globular clusters provides information crucial for the determination of the timescale on which the Galaxy formed and collapsed, on the assumption that the halo globular clusters formed during the collapse, and could not form once the bulk of the material had gone into the galactic disk. Did the Galaxy collapse on a purely dynamical timescale⁵, or was it slowed down by dissipation⁹? At what stage in the process did the individual globular clusters form?

Valuable constraints on cosmological parameters could be achieved with an accuracy in the absolute age of ~2 Gyr^{10,11}. For galactic studies, we note that a purely dynamical collapse from an outer radius of 50 kpc would take about 1 Gyr⁶. Thus, to discriminate between galactic evolution models one seeks a precision of 1 Gyr or better in relative age determinations.

Great advances have been made in the past decade in the accuracy with which globular cluster ages can be measured, through major developments in observational techniques and improvements in theoretical modelling. As a result of these developments, several claims of an age spread in the globular cluster system have been made^{12,13}, but all are subject to controversy^{14,15}. Much of the controversy arises from comparison of clusters of different metallicity; relative ages may be obtained with greater precision for groups of clusters with similar metallicities^{3,4}.

To this end, we concentrate our attention here on two globular clusters with similar metal abundances, NGC288 and NGC362, which have been extensively studied by others in an attempt to measure an age spread. For a number of years, NGC288 has been thought to be a prime candidate for an older-than-average cluster for two reasons. First, early photometric studies drew attention to the possibility that the 'turnoff' in the colour-

magnitude diagram (CMD), used to measure cluster age, might be considerably fainter than in other clusters. Second, the cluster seemed to be an extreme example of a cluster in which the 'second parameter' was operating¹⁶. It was known that heavy-element abundance (the 'first' parameter) was strongly correlated with the colour of 'horizontal-branch' (HB) stars, but some clusters seemed to violate the correlation, suggesting a second parameter was involved. The identity of this second parameter is still unknown, there being many candidates capable of fulfilling the role, including age; an older-than-average cluster of intermediate metallicity might be expected to have a purely blue HB in the CMD, as found in NGC288. The second cluster of the above pair, NGC362, has an extremely red HB; thus intensive study of this pair seems likely to throw light on both the second-parameter problem and the age spread. But in discussions of this problem, the presently arbitrary assumptions about mass loss in stars before they reach the HB add considerable uncertainty to any conclusions about which parameters are most important in controlling the HB morphology of actual clusters¹⁷.

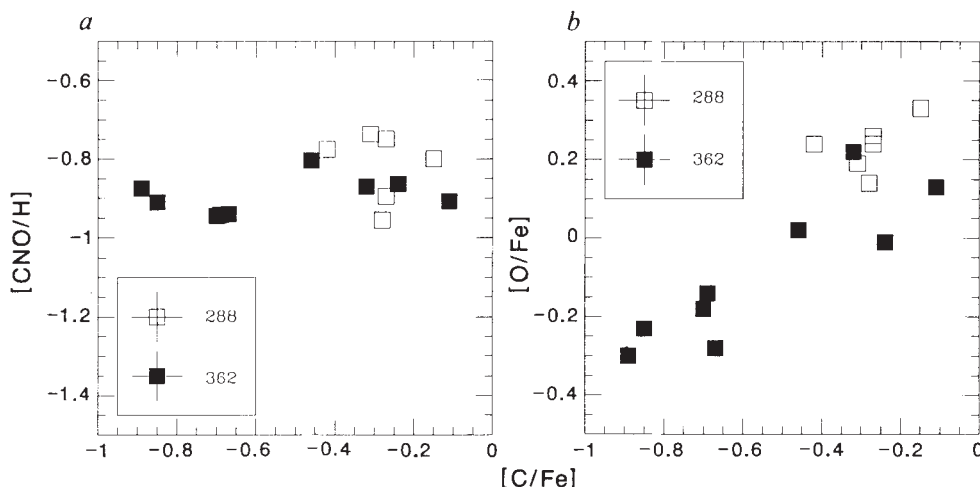
The recent attempts¹⁻⁴ on the age-spread problem have all claimed a small but significant NGC288/NGC362 age difference of 3 ± 1 Gyr^{1,2,4} or 2.5 ± 1.5 Gyr³, with NGC288 being the older. Of necessity these studies assumed that the abundances of C, N and O, which dominate the behaviour of stars at the critical turnoff region in the CMD, had the same ratios to heavy-element abundance in globular cluster stars as in the Sun. But an enhancement in oxygen (with respect to heavier elements) by a factor of three results in a decrease in age of ~2 Gyr^{7,8} from that derived for a given observed turnoff using isochrones of models with solar-abundance ratios.

To redress the situation, we have made measurements of the C, N and O abundances in a number of globular clusters, using a high-resolution echelle spectrograph (UCLES) on the Anglo-Australian Telescope (AAT), and analysis by spectrum synthesis. We have also obtained accurate heavy-element (Fe-peak) abundances (the 'first' parameter), also crucial for age determinations. We report first results for stars in NGC288 and NGC362, and discuss their implications for the age and second-parameter problems discussed above.

Spectral techniques yield abundance information for the stellar atmospheres, whereas the relevant quantities for age determinations, and other problems referred to above, are the interior values that influence the stellar structure and evolutionary timescales. The atmospheric composition will only represent the initial composition of the material from which the star formed if no mixing to the surface of interior products of nuclear burning has occurred. Evolved giants, which are the only globular cluster stars that are bright enough for a direct determination of the oxygen abundances, are known in some cases to have suffered considerable mixing from interior regions where H burning has modified the relative C, N and O abundances through CNO cycle reactions¹⁸. Thus, the possibility that individual surface abundances of C, N and O may not be representative of the primordial values must be taken into account. But the total (C+N+O) abundance is conserved in the CNO cycle, and that derived from evolved stars should indeed be the initial value of interest to us. Although of secondary consideration here, the individual abundances derived on the way to the total values must be understandable in terms of these physical effects (CNO cycle and mixing) if we are to assume confidently that they represent interior values.

C, N and O abundances have been obtained for six red giant stars in NGC288 and nine in NGC362, from observations of CH and CN molecular bands in the blue, and of the (forbidden) absorption line of [OI] at 6,300 Å in the red. Because the amount of free O available to form this line depends (through CO formation in the atmospheres of the cool stars we observed) on the abundance of C in the stellar atmosphere, simultaneous solution for C and O is required to determine the true O

FIG. 1 *a*, The total (C+N+O) abundance plotted against carbon abundance for red giant stars in NGC288 (open symbols) and NGC362 (filled symbols). Square brackets denote logarithmic abundance ratios relative to those in the Sun (see text). Typical errors in the final ratios are indicated by error bars in the boxed region. The diagram illustrates the near-constancy of the logarithmic abundance of CNO with respect to hydrogen, both within NGC362 (where the stars have a variable amount of mixing giving rise to the range in C-depletion shown) and between NGC288 and NGC362. The latter result demonstrates that a difference in CNO abundance cannot be responsible for the apparent age difference between the clusters, which is therefore believed to be real. *b*, The correlation between oxygen and carbon abundance, relative to iron, for red giants in NGC288 and NGC362, with definitions, symbols and error bars as



in (a). The strong correlation is expected as the result of material processed through the CNO cycle being mixed to the stellar surface.

abundance. This has been achieved through a full synthesis of all spectral regions, including complete treatment of the molecular equilibrium requirements, to obtain optimal fits to all the spectral features observed. The red observations were taken with UCLES at a spectral resolution of $\sim 35,000$ (full-width-half-maximum, FWHM, $0.2 \text{ \AA}$), and a signal-to-noise ratio per resolution element > 100 . Observations with UCLES in the blue had a similar resolution but a lower signal-to-noise ratio of ~ 20 . The data set was enhanced with a few earlier observations in the blue at lower resolution of $\sim 0.5 \text{ \AA}$ (FWHM). Fe and other elemental abundances have also been measured from the red UCLES spectra. Several standard stars¹⁹, together with Arcturus, have also been observed and analysed in the same way as the programme stars, to provide a thorough tie-in with previous work.

We find the heavy-element abundance, $[\text{Fe}/\text{H}]$, is almost identical in these two clusters: $[\text{Fe}/\text{H}] = -1.03 \pm 0.07$ for NGC288 and -0.98 ± 0.07 for NGC362. The square brackets denote logarithmic abundances by number of atoms relative to those in the Sun. Although these values are ~ 0.3 higher than those adopted in the above works cited¹⁻⁴, they are in good agreement with most other determinations²⁰, and our results for standard stars agree well with currently accepted values. In any case, it is the differential abundances that are of primary importance here. Our principal result is illustrated in Fig. 1*a*, in which the sum of the abundances of C, N and O with respect to H is shown plotted against the C abundance (relative to Fe) for the 15 stars studied. Mean values of $[(\text{C} + \text{N} + \text{O})/\text{H}]$ for each cluster are -0.82 ± 0.08 (NGC288) and -0.89 ± 0.05 (NGC362); the clusters have identical CNO abundances within the errors of determination.

We now consider briefly whether our results for the individual elements C, N, O and Fe are plausible. The results are conveniently illustrated in Fig. 1*b*, which shows $[\text{O}/\text{Fe}]$ plotted against $[\text{C}/\text{Fe}]$ for individual stars. The separate abundances in NGC362 correlate very strongly, as would be expected from material that has undergone CNO cycling, where both C and O are converted to N. This is indeed confirmed by strong anticorrelations found in our results between C and N, and between O and N. The stars in NGC288 behave differently, appearing not to have experienced much mixing of CNO material; they are similar to the least-mixed NGC362 stars. Clearly an additional mechanism, or another parameter, is needed to account for this difference between the two clusters, and for the variable amount of mixing between different stars

within NGC362. But the results are broadly as expected for variable amounts of mixing of CNO products to the surfaces of the stars, where ON as well as CN cycling has occurred, but the total (C+N+O) remains constant. The depletion in O by a factor of ~ 2 , shown by some of the NGC362 stars in Fig. 1*b*, is typical of that expected from partial equilibrium set up in the ON cycle²¹.

$[\text{C}/\text{Fe}]$ is typically near zero for halo dwarfs. Sneden²² finds $[\text{C}/\text{Fe}] \approx -0.2$ and suggests that the shift from zero is probably due to some systematic error. Thus, from Fig. 1*b* we see that $[\text{O}/\text{Fe}] \approx 0.2$ for unmixed stars. This would imply that $[(\text{C} + \text{N} + \text{O})/\text{H}]$ should be ~ 0.15 larger than $[\text{Fe}/\text{H}]$, just as we find. A full discussion of these aspects will be published elsewhere.

Examination of Fig. 1*a* and *b* also shows the possible danger in using a measurement of $[\text{O}/\text{Fe}]$ to infer a value of $[(\text{C} + \text{N} + \text{O})/\text{H}]$, the quantity of importance for determining stellar ages. Further, the fact that clusters with similar $[\text{Fe}/\text{H}]$ have such different mixing histories should raise a warning flag to those applying observations of $[\text{O}/\text{Fe}]$ from one sample of stars to infer global trends of $[(\text{C} + \text{N} + \text{O})/\text{H}]$ with metallicity. For a reliable cluster age, observations of C, N and O are required within that cluster. From our results, we see that non-solar ratios of these elements cannot introduce substantial error in the ages of NGC288 and NGC362. Most previous age determinations, both absolute and relative have, however, a built-in uncertainty of a few Gyr through lack of knowledge of the CNO abundance.

As far as the suggestion that NGC288 is 3 Gyr older than NGC362, the observed $\delta[(\text{C} + \text{N} + \text{O})/\text{H}]$ of 0.07 is about an order of magnitude too small to explain the observations by a difference in composition. We conclude that CNO plays only a minor role, if any, in the apparent age difference between these two clusters and that the clusters indeed differ in age by ~ 3 Gyr. Furthermore, in support of earlier claims²⁰, CNO abundance is clearly not the second parameter.

Can a rapid collapse model of the Galaxy therefore be ruled out? As a dynamical timescale could be as long as 1 Gyr, or even longer, and this is within $1-2\sigma$ of the results cited above, we cannot yet exclude the possibility that the region containing these two clusters collapsed on a dynamical timescale. This is at first sight disappointing, in that the formal error, even in differential age determinations with complete abundance data, still seems to be too large to provide a conclusive result for these clusters. But there is additional evidence that differential methods may eventually yield useful results. First, for a group of very metal-poor clusters, VandenBerg *et al.*³ conclude that

there is no convincing evidence for an age spread >0.5 Gyr, with an intrinsic age dispersion of ± 0.2 Gyr. Both the age difference, and its uncertainty, are nearly an order of magnitude smaller than those found for NGC288/NGC362. The small formal error presumably results from higher-quality data, and better-known fitting parameters (for example, reddening). The derived age difference, however, depends on their assumption that the clusters have the same (including CNO) abundances. An almost identical and essentially independent result was obtained by van Albada *et al.*⁶ from analysis of ultraviolet. Astronomical Netherlands Satellite photometry of a sample of very metal-poor clusters (which include several considered by VandenBerg *et al.*³) in which they concluded that a firm upper limit for the dispersion of the distribution of ages was 0.3 Gyr.

As the age spread found by van Albada *et al.*⁶ also depends on assuming solar CNO/Fe abundances, and uses similar stellar models, we believe this agreement to be significant. Second, the method²³ of matching the whole CMD with theory holds great promise, because our results nicely corroborate the implication from the NGC288 and NGC362 CMDs² that a large difference in abundance is unlikely. Thus, with accurate abundances of the relevant elements, and with relatively small improvements in the precision of the data, and parameters involved in the fitting, more definitive results could be within reach for clusters such as the NGC288/NGC362 pair. Already, we feel that the above results are not easily discounted and a more complex picture of early Galaxy formation and evolution than that envisaged in the simple rapid collapse model is required. $\square$

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A hot gas model for iron-line X-ray emission from the rapidly varying Seyfert galaxy NGC6814

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KUNIEDA *et al.*¹ have observed rapidly varying X-ray continuum emission, along with an emission line at 6.4 keV, from the Seyfert I galaxy NGC6814. They suggest that a central energy source of radius $\sim 10^{12}$ cm generates X-rays that produce the emission line by fluorescence of iron in a surrounding cold gas, which extends out to $\sim 10^{13}$ cm. But in this model the intense X-irradiation needed to explain the line intensity would heat the surrounding gas to too high a temperature to allow the existence of the low ionization state needed to account for the wavelength of the line. I argue here, therefore, that the line is generated by hot gas, closer to the central energy source, and that the observed wavelength corresponds to the energy of a higher ionization state of iron, gravitationally redshifted by $\sim 6\%$.

Kunieda *et al.*¹ observed X-rays in the energy range 2–20 keV from the Seyfert I galaxy NGC6814 and found rapid variations of continuum X-rays (the shortest timescale, t , was 50 s) and of the iron emission line. The variations were synchronized within a time resolution of 256 s. From the timescale of variability, t , they obtain the maximum size of the emission region, which must be smaller than the distance travelled by light in this time, ct , which is less than 1.5×10^{12} cm for the continuum X-rays. Because X-rays are supposed to be powered by accretion of matter onto a massive black hole of mass M , the continuum X-ray emission region lies outside the last stable orbit of radius $3R_g$, most effectively in the region around $5R_g$, where $R_g = 2GM/c^2$ is the Schwarzschild radius. Kunieda *et al.*¹ assumed $5R_g \leq 1.5 \times 10^{12}$ m and therefore $M \leq 10^6 M_\odot$.

The energy of the iron emission line¹ was ~ 6.4 keV after a small cosmological redshift correction ($z_c = 0.0053$). Such a line has been observed for most Seyfert galaxy sources and interpreted as a result of the fluorescence of iron irradiated by continuum X-rays. The energy of 6.4 keV indicates that iron in low ionization states is responsible for this Fe K line. The line-emitting matter at a radius R rotates around the black hole at a speed of $c(R_g/2R)^{1/2}$, so that the line would be broadened by a Doppler effect depending on the view angle. As the observed line width was <1 keV, Kunieda *et al.*¹ assumed the face-on geometry and gave $R \approx 25R_g$, to be consistent with the variability timescale (<256 s) and an unappreciable redshift. The redshift arises from gravitational and relativistic effects, according to which the observed energy, E , is related to the emission energy E_0 by

$$E = E_0 \left(1 - \frac{R_g}{R}\right)^{1/2} \left(1 - \frac{R_g}{2R}\right)^{1/2} \quad (1)$$

This interpretation is based on a widely accepted model of Seyfert I X-ray sources, in which continuum X-rays emitted from a compact central region irradiate surrounding matter located at a rather large distance, say 1 pc, to produce fluorescence lines at a temperature $<10^6$ K. Krolik and Kallman² worked out the Fe K features for this model and predicted an equivalent width (the line intensity divided by the continuum intensity per energy) of <120 eV for the $K\alpha$ line and a K-edge opacity large enough to be observable, if the surrounding matter forms a torus of height $H \approx 0.7R$. The equivalent width observed, however, was 300–360 eV, and the K-edge absorption was insignificant¹. These Fe K features and the rapid variability indicate that NGC6814 is a unique object and requires a different model.

In the earlier model¹, it is difficult to keep the ionization state of iron low. The X-ray luminosity in the bright phase reached $\sim 10^{43}$ erg s⁻¹ in the range 2–20 keV with a Hubble constant of 50 km s⁻¹ Mpc⁻¹. The luminosity of the ionizing radiation, L_{ion} ,

may be as high as $2.5 \times 10^{43} \text{ erg s}^{-1}$ (ref. 3). At a distance of $R = 10^{13} \text{ cm}$, as in ref. 1, we expect a high ionization parameter^{4,5} $\xi = L_{\text{ion}}/nR^2$ or $\Xi = L_{\text{ion}}/4\pi R^2 c n k T$, where n and T are the number density and temperature of matter in the line-emission region. Iron can remain in low ionization states only if $\xi \leq 30 \text{ erg cm s}^{-1}$ or $\Xi \leq 10$; these critical values slowly decrease as the radiation spectrum becomes softer⁶. The interpretation of ref. 1 would be possible only if $n \geq 10^{16} \text{ cm}^{-3}$ and $T > 5 \times 10^4$. This density is too high, as will be seen later.

I therefore propose a different model, in which both the continuum and iron lines are produced in roughly the same region at a distance R from the centre. Because of a high continuum flux and an additional heating process discussed later, matter in this region is highly ionized, and the Fe K line arises from H-like and He-like ions with a mean energy of $E_0 \approx 6.8 \text{ keV}$. This is redshifted to 6.4 keV according to equation (1). Thus we obtain $R/R_g \approx 13$, or

$$R \approx 3.8 \times 10^{12} M_6 \text{ cm} \quad (2)$$

where M_6 is the mass of the central black hole in units of $10^6 M_\odot$. As R/R_g is small, any deviation from face-on geometry should be small if the Doppler shift is to be negligible. A 50-s variation occurs for $M_6 \approx 0.5$, which corresponds to an Eddington luminosity of $\sim 8 \times 10^{43} \text{ erg s}^{-1}$, comparable to the bolometric luminosity of $\sim 7 \times 10^{43} \text{ erg s}^{-1}$ estimated in ref. 3.

The line width of $<1 \text{ keV}$ indicates that the emission region forms a torus with its axis nearly parallel to the line of sight and a minor radius of $H \leq 0.5R$. Matter in the torus with an average density n falls inward with an infall velocity v and is accreted by the central black hole, thus powering X-rays with a luminosity of $\sim L_{\text{ion}}$. The luminosity is proportional to the accretion rate $\dot{M}$:

$$L_{\text{ion}} = \dot{M} c^2 \eta = 4\pi R H n m_H v c^2 \eta \quad (3)$$

where the infalling matter is assumed to consist of hydrogen with atomic mass m_H . Equation (3) gives the average density

$$n \approx 3 \times 10^{12} \left(\frac{L_{\text{ion}}}{2.5 \times 10^{43} \text{ erg s}^{-1}} \right) \times \left(\frac{H/R}{0.5} \right)^{-1} \frac{1}{v_{10} R_{12}^2 \eta_1} \text{ cm}^{-3} \quad (4)$$

where v_{10} , R_{12} and η_1 are the values of v , R and η in units of $10^{10} \text{ cm s}^{-1}$, 10^{12} cm and 10^{-1} , respectively.

Several observations suggest that radiating matter in active galactic nuclei is clumped to form clouds of density n_{cl} with a radius r and a filling factor $f = n/n_{\text{cl}}$ (see, for example, ref. 3). These clouds are heated by the Compton scattering of continuum X-rays to several keV if the heating is balanced by radiation cooling for $n_{\text{cl}} \approx 10^{15} \text{ cm}^{-3}$. Hence iron may be ionized highly enough to emit H- and He-like K lines. The line emissivity is estimated⁶ as $\sim 5 \times 10^{-25} \text{ erg cm}^3 \text{ s}^{-1}$. The observed line luminosity of $2.5 \times 10^{41} \text{ erg s}^{-1}$ requires the emission measure

$$\text{EM} = 2\pi^2 R H^2 n_{\text{cl}}^2 f = 5 \times 10^{65} \text{ cm}^{-3} \quad (5)$$

which gives a small fraction of continuum X-rays. This gives

$$n_{\text{cl}} = n/f = 1 \times 10^{14} \left(\frac{H/R}{0.5} \right)^{-1} R_{12}^{-3/2} f^{-1/2} \text{ cm}^{-3} \quad (6)$$

So that the lines are observable without being smeared by Compton scattering, the cloud radius r is limited by $r n_{\text{cl}} \leq 10^{24} \text{ cm}^{-2}$. On the other hand, the clouds should not overlap with each other when viewed face-on, thus requiring $(3/4)(H/r)f < 1$. These two conditions result in

$$3.7 \times 10^{11} f \leq r \leq 1.4 \times 10^{10} f^{1/2} \quad (7)$$

for the value of R obtained with $M_6 = 0.5$ in equation (2) and $H/R = 0.5$. This is satisfied for $f^{-1} \geq 7 \times 10^2$ and accordingly

$r \approx 10^9 \text{ cm}$. Substituting $f^{-1} = 7 \times 10^2$ in equation (6), we obtain $n_{\text{cl}} \approx 1 \times 10^{15} \text{ cm}^{-3}$ and $n \approx 1.4 \times 10^{12} \text{ cm}^{-3}$. The latter is consistent with equation (4) if the infall velocity $v = 10^{10} \text{ cm s}^{-1}$ and $\eta_1 = 0.5$.

The collision of clouds would have a large effect if their random velocity v_r were high. The collision time of r/v_r can be shorter than the infall time R/v only if v_r is greater than $5 \times 10^8 \text{ cm s}^{-1}$. Such a high velocity generates a shock wave to heat the clouds to a temperature much higher than that appropriate for the line emission. It is therefore likely that the random velocity is so small that cloud collisions have little effect.

I have determined a set of parameters that are consistent with a model describing the emission of continuum and Fe K-line X-rays from a toroidal region with a major radius of $\sim 2 \times 10^{12} \text{ cm}$. This model differs from that adopted for most Seyfert galaxies, thus indicating the uniqueness of NGC6814, which is probably due to the small mass of the central black hole. To implement the model, it will be necessary to study dynamical behaviour of the accretion flow and the generation of continuum X-rays, but this is beyond the scope of the present paper. $\square$

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Observation of surface reconstruction on silicon above 800 °C using the STM

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AN understanding of the kinetics of structural phase transitions in crystalline, semiconducting thin films is important for the control of film growth in techniques such as molecular-beam epitaxy, as well as in applications of such films at high temperatures. Electron diffraction techniques (LEED and RHEED) have been applied to the study of high-temperature surface reconstruction, but although these can detect changes in surface periodicity, they do not permit atomic-scale resolution of the nucleation and growth processes. The scanning tunnelling microscope (STM) provides an alternative tool to investigate changes of this sort, but high-temperature observations have been hampered by problems of thermal drift. Here we report our success in overcoming these problems and thus in obtaining atomic-resolution STM images of the $1 \times 1 \rightarrow 7 \times 7$ surface reconstruction of a silicon thin film at about 860 °C. Step formation and migration are clearly visible in these images.

Since Binnig *et al.*¹ achieved atomic scale imaging of the 7×7 reconstruction on the surface of Si(111) with the scanning tunnelling microscope (STM), it has been used increasingly for surface analysis. There is also an increasing need to observe samples under ultra-high-vacuum (UHV) conditions and at high temperatures. High-temperature measurements can provide information on the dynamics of thin-film development and surface phase transitions. Most studies of this sort have, however, involved first heating the sample but then cooling it to room temperature before STM imaging; observations are rarely made while the film is being heated. Atomic-scale STM

imaging at high temperatures is difficult for a number of reasons, among them drift of the sample, stage and STM tip, transfer of vibrations through electrical leads and thermal damage to piezoelectric elements. Nevertheless, an image of the 5×5 structure of Si(111) at 320 °C has been obtained by Feenstra *et al.*²

In general, clean surfaces of a semiconductor show structures different to that of the bulk (reconstruction structures). The 7×7 reconstruction formed on a Si(111) surface, for example, has a unit cell seven times larger than that of the 1×1 bulk structure. As the temperature of Si(111) is lowered below 830 °C, it undergoes a phase transition⁴ from the 1×1 to the 7×7 structure. This transformation has been verified by LEED and RHEED^{3,4}. The dynamics of the phase transition have been studied using UHV-REM (reflection electron microscopy)⁵, UHV-TEM (transmission electron microscopy)⁶ and UHV-LEERM (low-energy electron reflection microscopy)⁷. These methods, however, can only provide images of changes in the sizes of 1×1 and 7×7 domains, and are unable to resolve structural changes on the atomic scale.

We have used a UHV STM (JSTM-4000XV)⁸ that is designed for studying heated samples. The vacuum pressure in the viewing chamber of this instrument can be less than 2×10^{-9} Pa. We used n-type, p-doped Si with a resistivity of 2–3 Ω cm, oriented within 1° of the (111) plane. The sample was cut into 1×7 mm strips 0.3 mm thick, which were cleaned ultrasonically in pure acetone. A strip was then placed in the ultra-high-vacuum chamber and kept at 450 °C overnight, then heated to 900 °C by an electric current of 0.9 A while the vacuum pressure was maintained at $\leq 2 \times 10^{-8}$ Pa. An electrolytically polished tungsten wire (0.3 mm diameter) was used as the STM tip.

The sample was first observed at room temperature to verify that the 7×7 reconstruction had taken place and that the surface was clean. It was then heated to 1,250 °C by thermal flashing, and the temperature was decreased to close to that of the phase transition, at which point STM imaging was carried out. The bias voltage at the centre of the sample, where imaging took place, was estimated as the average of those at each end of the sample. The sample temperature was measured by an IR-AP digital infrared-radiation thermometer from Chino Co.

(measured wavelength of 0.96 μ m, rated accuracy of $\pm 0.5\%$). The average temperature of the 0.8-mm-diameter imaged region of the sample was measured through the viewing port on the basis of a sample emissivity of 0.43.

Figure 1 shows a series of STM images obtained close to the phase transition temperature (T_c) of 860 °C: Fig. 1a, b were obtained at 880 °C, Fig. 1c at 860 °C and Fig. 1d at 840 °C. Figures 1a and b are respectively constant-current (topographic) and current images of the same area at the same temperature, b being taken 30 s after a. As the atomic corrugation in the constant-current image (Fig. 1a) is smaller than the step height between the terraces, this image loses atomic resolution. Figure 1b on the other hand, acquired by increasing the time constant of the feedback circuit to allow for the tilt of the entire sample, is able to observe terraces while maintaining atomic resolution. Between Fig. 1a and b, the steps have moved slightly. The tip scans from left to right in these images, so that when the terraces are descending towards the right, as in Fig. 1b, steps are represented as dark lines because the tip moves away momentarily from the step as a result of the large time constant of the feedback circuit. Similarly, when terraces ascend towards the right, the steps appear white.

Each time the temperature was lowered (Fig. 1a, b to c, and c to d), a period of 30 min was allowed between imaging so that sample drift became stabilized. In the image at 860 °C (Fig. 1c), the 7×7 reconstruction is completed on the upper side of the step, whereas atoms are seemingly in motion on a bulk-like 1×1 layer near the lower side of the step. We verified that the position of this step was in motion.

The 840 °C image (Fig. 1d) shows that the surface of the sample is now covered with the 7×7 reconstruction, and that the step is aligned with the orientation of a 7×7 unit cell, [011]. The image also demonstrates that the step is still advancing or retreating by one or a few 7×7 unit cells; for example, the step has moved by one unit cell from point A to point B. It is not clear, however, whether or not the entire step is advancing.

Figure 2a–c shows STM images of identical areas taken at temperatures of 864, 857 and 854 °C respectively. The arrowheads indicate steps. These images were all taken within 90 s,

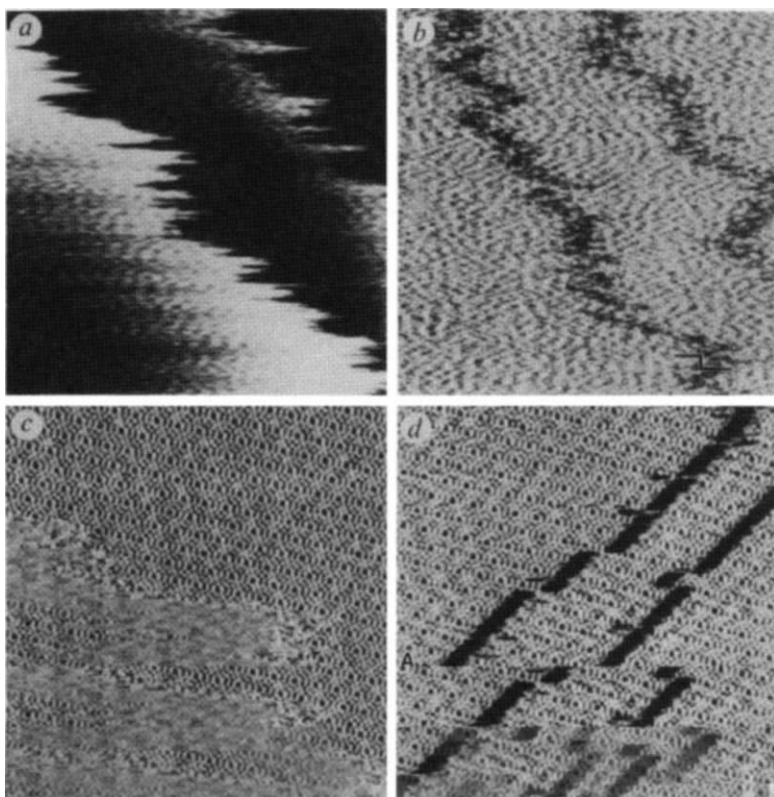
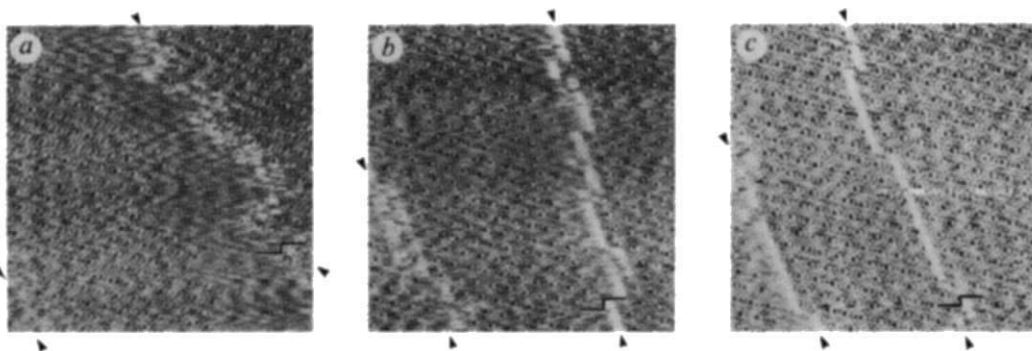


FIG. 1 STM images of heated Si (111) surface. a, Constant-current image at 880 °C. b–d, Current images at 880 °C (b), 860 °C (c), 840 °C (d). Scanned area is 100×100 nm for a and b, and 50×50 nm for c and d. Sample bias voltages are +1.6 V for a and b, +1.5 V for c and +2.0 V for d.

FIG. 2 *a*, Current of Si(111) close to the surface transition temperature. *a*, 864 °C. Sample bias voltage +1.95V. *b*, 857 °C. Sample bias voltage = +1.98V. *c*, 854 °C. Sample bias voltage = +2.00V. Scanned area is 50 × 50 nm in all cases.



and demonstrate that there were no problems with thermal drift. These images show the development of the 7 × 7 reconstruction from the upper side of the step as temperature decreases. At 854 °C the entire centre terrace has acquired the 7 × 7 structure. The step is aligned with the $[\bar{1}10]$ orientation of the 7 × 7 unit cell. As this structure has three possible orientations, steps may become aligned in any of three different directions. Osakabe *et al.*⁸ have reported that step edges are smooth when $T > T_c$ but zigzagged when $T < T_c$. It seems that the zigzag shape may be due to steps growing in the three different directions ($[\bar{1}10]$, $[\bar{1}01]$ and $[0\bar{1}1]$).

Although LEED and RHEED results^{3,4} indicate that the 1 × 1 structure is formed above T_c , these techniques provide only information on the bulk. We were not able to resolve the surface structure at such temperatures in our images, presumably because the surface atoms were thermally excited. Nevertheless, we were able to reduce thermal drift rates in these experiments to 0.05 nm s⁻¹.

STM imaging at high temperatures offers a great deal of local information. This kind of information on surface properties is needed to improve absorption characteristics and ion-implantation processes, as well as for high-temperature device fabrica-

tion. Stable STM imaging at high temperatures was made possible here for the following reasons: (1) the specimen stage was designed so that thermal diffusion takes place concentrically. (2) The sample size was minimized to reduce heat dissipation. (3) Because of the low heat dissipation, current flowing to the sample was reduced and vibration from the electrical leads was minimized. (4) Radiation from the heating sample to the tip and piezoelectric elements was also minimized. The scanning speed of this STM is currently 10–20 s per frame for a 50 × 50 nm area, precluding observation of sporadic or sudden atomic movement. This is a problem for the future. □

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Highly vibrationally excited oxygen as a potential source of ozone in the upper stratosphere and mesosphere

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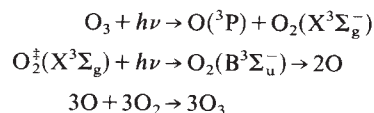
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ONE of the main problems in atmospheric chemistry in recent years has been the discrepancy between theoretical model calculations of ozone concentrations and observations in the upper stratosphere and mesosphere¹. It has been suggested² that the photolysis of vibrationally excited oxygen could provide an extra source of ozone and new laboratory data now allow us to test this hypothesis in two ways. First, by including the proposed mechanism in a numerical model of the atmosphere, we find that the production of odd oxygen is enhanced and that the calculated ozone concentrations are significantly increased so that they agree well with observations. Second, we present a comparison between satellite observations of the daytime enhancement in the 6.9-μm emission from water vapour and theoretical calculations, which yields indirect evidence for the presence of vibrationally excited oxygen in the upper stratosphere and mesosphere; a significant fraction of which appears to be due to relaxation of very high vibrational

levels. Both these tests demonstrate that the proposed mechanism may indeed account for most of the discrepancy between model results and observations.

Theoretical model studies^{3–5} have made use of large satellite data sets to investigate the atmospheric ozone budget. The models underestimated upper stratospheric ozone concentrations by 30–50%. A more recent study⁶ concluded that the constrained model calculations of ozone up to altitudes of 50 km agree with observations to within 22%, but are nevertheless consistently lower at all altitudes. At altitudes of 50–80 km, Rusch and Eckman⁷ concluded that calculated ozone densities were lower by up to a factor of two.

The mechanism considered in this paper was proposed by Slanger *et al.*² and involves the photodissociation of vibrationally excited $O_2(X^3\Sigma_g^-)$, O_2^+ , generated by the triplet channel in the Hartley band:



Slanger *et al.* concluded that the mechanism could be of atmospheric importance if high vibrational levels ($v'' > 15$) are populated.

Dissociation of ozone at wavelengths less than 300 nm produces four species:

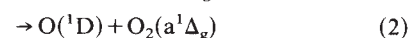
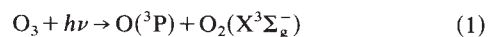


TABLE 1 Model calculations for 30°N, September, noon and for $v''=15$				
Altitude (km)	T (K)	$10^{-16} k_{v''}$ (molecules $\text{cm}^{-3} \text{s}^{-1}$)	$10^{-2} J_{v''}$ (10^{-2}s^{-1})	O_2^+ (10^6 molecules cm^{-3})
58	240	6.0	3.5	3.1
55	253	7.8	3.5	3.1
51	265	9.8	3.5	2.7
47	264	9.6	3.4	3.0
43	258	8.6	3.4	2.7
39	252	7.6	3.4	1.7
36	240	6.0	3.4	1.2

The quantum efficiency of process (1) is ~ 0.13 , and there is sufficient surplus energy for vibrational excitation^{8,9} of O_2 ($X^3\Sigma_g^-$). A detailed vibrational distribution has recently been derived¹⁰ by the technique of ozone fragmentation at 226 nm. This new information allows us to estimate the importance of the mechanism proposed by Slinger *et al.*²

We have used the vibrational distribution, assumed constant with altitude, in a photochemical model¹¹ to assess the importance of O_2^+ as a source of ozone. The photolysis of vibrationally excited oxygen produces two molecules of odd oxygen. We calculate the total production rate of odd oxygen, P_{Ox} , as the sum of the production from all levels up to $v''=21$.

$$P_{\text{Ox}} = 2 \sum_{v''=1,21} J_{v''} [\text{O}_2^+]_{v''}$$

where $J_{v''}$ is the photolysis frequency for O_2 in level v'' and $[\text{O}_2^+]_{v''}$ is the concentration of O_2^+ in level v'' .

The computation of $J_{v''}$ requires absorption cross-sections and the photon flux at a certain altitude. We have followed the procedure of Slinger *et al.* and scaled the product of frequency and Franck-Condon factor for a transition¹² to calculate absorption cross-sections of O_2^+ for the bands $v'=0-13$, $v''=1-21$. The photolysis coefficient for a given v'' is then calculated by summing the product of the absorption cross-section, $\sigma_{v',v''}$, and the incident flux, q , in the wavelength range $\lambda = 176-480$ nm.

$$J_{v''} = \sum_{\lambda} (\sum_{v'=0,13} \sigma(\lambda, v', v'') q(\lambda))$$

The concentration of O_2^+ in any vibrational level v'' depends on the direct photochemical production by the photolysis of ozone and on the quenching of O_2^+ by O_2 in the level above, $v''+1$, as

TABLE 2 Model calculations for 30°N, September, noon					
Altitude (km)	$2J_2\text{O}_2$ (10^6 molecules $\text{cm}^{-3} \text{s}^{-1}$)	P_{Ox} (10^6 molecules $\text{cm}^{-3} \text{s}^{-1}$)	%*	O_3 (p.p.m.v.) A B	%†
58	4.6	2.8	60	1.5 2.0	37
55	5.5	3.2	57	1.9 2.5	35
51	6.9	3.0	43	2.3 2.9	28
47	9.0	3.5	39	3.1 4.0	26
43	11.1	3.3	29	4.4 5.2	20
40	12.2	2.2	17	5.8 6.4	11
36	11.6	1.3	13	7.7 8.1	5

A is the control run, B the run including the photolysis of O_2^+ .
* Per cent increase in odd-oxygen production.
† Per cent increase in ozone.

these mechanisms produce O_2^+ . Assuming steady state,

$$[\text{O}_2^+]_{v''} = \frac{\phi 0.13 J_1 [\text{O}_3] + k_{v''+1} [\text{O}_2] [\text{O}_2^+]_{v''+1}}{k_{v''} [\text{O}_2] + J_{v''}}$$

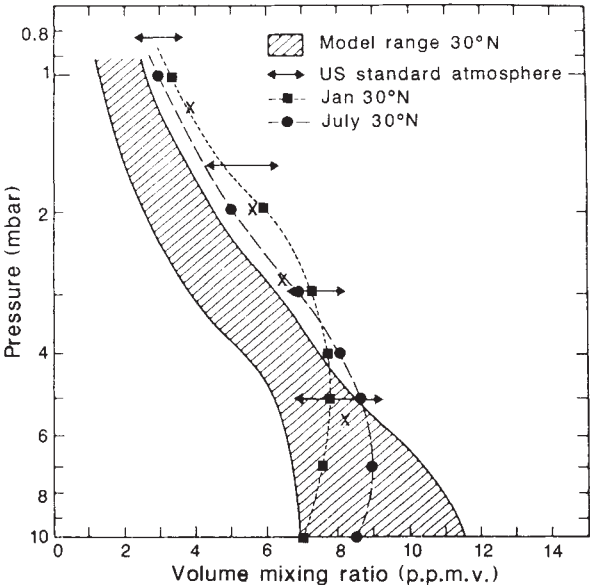
where ϕ is the quantum efficiency for level v'' (ref. 10), 0.13 is the quantum efficiency of the triplet channel of ozone photolysis in the Hartley band, J_1 is the photolysis coefficient of ozone in the Hartley band, $[\text{O}_3]$ is the concentration of ozone, $k_{v''+1}$ is the quenching rate constant at level $v''+1$, $[\text{O}_2^+]_{v''+1}$ is the concentration of O_2^+ in level $v''+1$, $k_{v''}$ is the quenching rate constant at level v'' , and $J_{v''}$ is the photolysis coefficient at level v'' .

The vibrationally excited states of O_2^+ will be mainly quenched ($v''=n \rightarrow v''=n-1$) by an interchange of vibrational energy with O_2 ($0 \rightarrow 1$). An equation for the probability of the vibration-to-vibration state transfer for ($1 \rightarrow 0/0 \rightarrow 1$) has been presented by Rapp¹³, and can be thought of as a first approximation for ($v''=n \rightarrow v''=n-1/0 \rightarrow 1$) transfer¹⁴. The quenching rate constant is then calculated as the product of the probability, oxygen collision cross-section and mean velocity. The probability, P , varies strongly with v'' . For example, for $v''=16$ we find $P=10^{-6}$ whereas for $v''=8$ we find $P=10^{-4}$.

Photolysis coefficients, quenching rate constants and $[\text{O}_2^+]$ were calculated for $v''=1-21$. Table 1 shows values for $v''=15$ as an example. Excited oxygen in the higher vibrational states is photolysed at longer wavelengths where the incident flux is also larger, and the photolysis coefficient thus increases with v'' .

Table 2 shows model results for two runs: a control run (A) and a run including the photolysis of O_2^+ (B). In the control run

FIG. 1 Upper-atmosphere ozone mixing ratio. Model results including the photolysis of vibrationally excited oxygen (x). The plot also shows the results of two-dimensional models (shaded region) for winter and summer, US standard atmosphere (horizontal arrows) and representative observations in January (■) and July (●) at 30°N, all as taken from WMO¹.



the only source of odd-oxygen production is the photolysis of unexcited O_2 , tabulated as $2J_2O_2$. Also shown is the rate of odd-oxygen production by photolysis of O_2^+ , $P_{O_2^+}$. Above 32 km there is a significant increase (60% at 58 km) in the total rate of odd-oxygen production when the new mechanism is included. The corresponding increase in ozone is also listed in Table 2. The percentage increase in ozone concentration (37% at 58 km) is not equal to the percentage increase in the rate of production, because the concentration of $O(^3P)$ increases significantly so that the rate of loss of odd oxygen (ozone) also increases. Below 36 km no significant enhancement was calculated by the model.

The model results for the upper stratosphere are plotted in Fig. 1, which shows the discrepancy between calculations of the vertical distribution of the ozone mixing ratio by a range of

two-dimensional models¹. The agreement between the model and observations is now substantially improved by the inclusion of the photolysis of O_2^+ . More important than the absolute agreement, which is sensitive to temperature, chlorine monoxide and so on, is the demonstration that the mechanism can be responsible for significant ozone production. The daytime enhancement in 6.9- μm emission in the upper stratosphere and mesosphere measured by the limb infrared monitor of the stratosphere (LIMS)¹⁵ is also consistent with the presence of O_2^+ .

Several mechanisms have previously been postulated to explain the enhanced emission¹⁶. Figure 2 shows calculations including or neglecting emission from $H_2O(010) \rightarrow (000)$ caused by photochemical production of O_2^+ , followed by relaxation to $O_2(v''=1)$ and vibration-to-vibration transfer to $H_2O(010)$. Four different processes are considered for generation of O_2^+ (see legend of Fig. 2), and agreement with measurement is generally improved when these are included. We therefore believe this to be indirect evidence for vibrationally excited oxygen in the upper stratosphere and mesosphere. Including process (1) significantly increases the photochemically induced component (by 50% at 65 km), although uncertainties in both theoretical calculations and measurements are too large for the effect of $O_2(v'' > 15)$ to be distinguished from that of lower levels which are also pumped by other processes.

The upper atmosphere research satellite (UARS) includes two instruments that should permit a more stringent test. First, ozone measurements will be made by the microwave limb sounder at millimetre wavelengths, where atmospheric emission is expected to be strictly thermal. Second, the improved stratospheric and mesospheric sounder will be very selective to emission from $H_2O(010)$. For a definitive test, new laboratory measurements will also be needed, especially to determine the fraction of electronic energy converted to vibrational energy in the quenching of $O_2(a^1\Delta_g)$ and $O(^1D)$ by O_2 and the rate of collisional relaxation of $O_2(v''=1)$ by O at atmospheric temperatures.

A number of uncertainties, which are difficult to quantify, also remain in the theoretical calculation of ozone concentrations, including quenching rates and the wavelength dependence of vibrational quantum yields as a function of altitude. As most of the ozone will be photolysed at wavelengths greater than 226 nm, the ozone production calculated here must be considered as an upper limit. Nevertheless, we believe that the evidence presented here is sufficient to indicate that vibrationally excited oxygen is present in the upper stratosphere and mesosphere and merits further consideration as a source of ozone in this region. □

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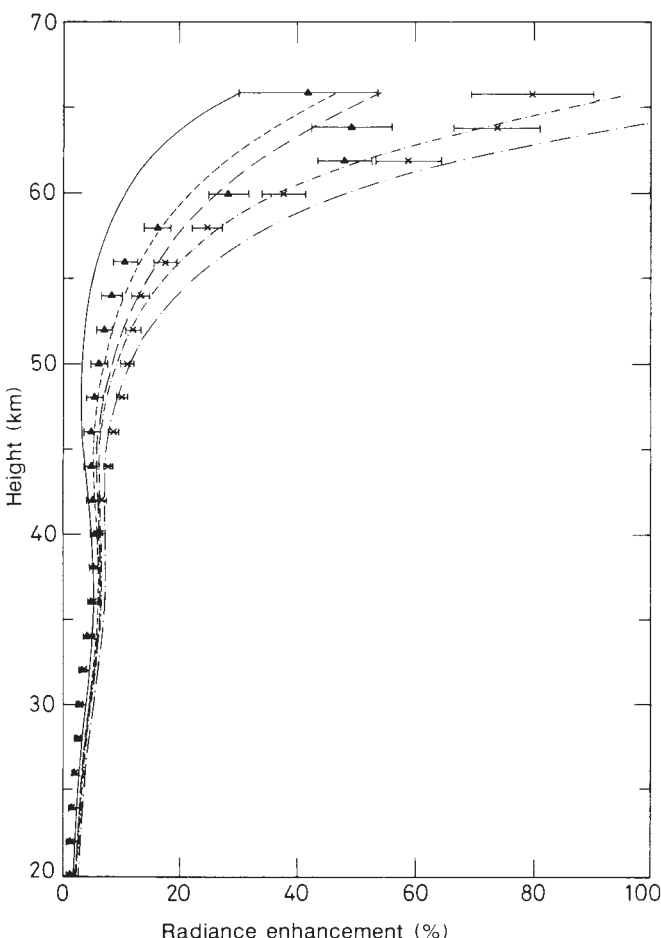


FIG. 2 Comparison of theoretical calculations with the daytime enhancement of 6.9- μm emission as measured by LIMS. Crosses and triangles with error bars: daytime enhancement derived by two different procedures (see ref. 16) from LIMS measurements. Solid line: calculated daytime enhancement due to processes other than excited O_2 photochemistry (see ref. 16). Short-dashed line: as for solid line, but also including the pumping of $H_2O(010)$ caused by photochemical production of vibrationally excited O_2 in the ($X^3\Sigma_g^-$) state by (1) Huggins and Chappuis band photolysis of O_3 (ref. 17) and (2) collisional quenching of $O_2(a^1\Delta_g)$ by $O_2(X^3\Sigma_g^-)$ (ref. 18) and in the ($a^1\Delta_g$) state mainly through the channel of Hartley band photolysis of O_3 (ref. 19). We adopt the temperature extrapolation of Lopez *et al.*²⁰ for O relaxation of $O_2(v''=1)$ and correct the LIMS O_3 profile for deviations from local thermodynamic equilibrium²¹ at 6.9 μm . Long-dashed line: as for short-dashed line, but including additional pumping of $H_2O(010)$ due to photochemical production of highly vibrationally excited $O_2(X^3\Sigma_g^-)$ through process (1) (ref. 10). Dot-and-short-dashed line: as for short-dashed line but adopting the Taylor²² temperature extrapolation for O relaxation of $O_2(v''=1)$ and not correcting the LIMS O_3 profile for deviation from local thermodynamic equilibrium. Dot-and-long-dashed line: as for dot-and-short-dashed line but including additional pumping of $H_2O(010)$ due to photochemical production of highly vibrationally excited $O_2(X^3\Sigma_g^-)$.

Evidence for lower productivity in the Antarctic Ocean during the last glaciation

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BOTH increased biological productivity and more efficient uptake of upwelled nutrients in high-latitude oceans have been proposed^{1–5} as mechanisms responsible for the glacial reduction in atmospheric concentrations of carbon dioxide deduced from ice-core measurements^{6–8}. These glacial models invoke more efficient 'biological pumping' of carbon into the deep sea by increasing the uptake of 'excess' biolimiting nutrients in the Antarctic surface ocean⁹ or by reorganizing chemical circulation patterns within the ocean^{10,11}. Here we challenge this conventional view with new evidence from tracers of palaeoproductivity preserved in Antarctic sediments. Records of the accumulation rates of diatom shells, the ratio of germanium to silicon in diatomaceous opal and the carbon isotope ratio in foraminiferal carbonate all suggest lower glacial productivity and less efficient uptake of nutrients. Although alternative interpretations are possible, our results support previous studies that indicate lower glacial productivity in the Southern Ocean^{12,13} and raise new questions about the role of ocean productivity in models of the causes (or remedies) for changes in atmospheric concentrations of carbon dioxide.

Eleven piston cores forming a north-south transect across the present Polar Front Zone (PFZ, at ~50° S) in the South Atlantic Ocean (Table 1) were analysed for opal at 5–20 cm intervals¹⁴. (Ge/Si)_{Opal} was determined on separated and purified diatoms in the <38 µm size fraction¹⁵. The δ¹⁸O and δ¹³C of the planktonic foraminifera *Neoglobobulimina pachyderma* l.c. were measured with a Finnegan MAT 251 mass spectrometer equipped with an automated carbonate preparation device¹⁶.

Using planktonic and/or benthic foraminifera δ¹⁸O records for seven of these cores¹⁷, we selected depth intervals corresponding to isotopic events 2.0 (12 kyr), 3.0 (24 kyr), 4.0 (59 kyr), 5.0 (74 kyr) and 6.0 (129 kyr)¹⁸. These horizons were extrapolated to cores lacking δ¹⁸O records by visual correlation to two well-dated reference cores (RC13-271, RC15-93) via *Cycladophora davisiana* abundances¹⁹ and %opal data. Additional isotopic events and the extinction level of *Stylatractus universus*²⁰ (isotopic event 12.0)²¹ were identified in RC13-259 (ref. 17). Our age models assume zero age for tops of cores containing a well-defined Holocene and constant sedimentation rates between isotopic events. The δ¹⁸O and δ¹³C records for cores in Table 1 are published elsewhere^{16,17}.

In sediments north of the present PFZ, biogenic opal contents are lower in the Holocene than during the Last Glacial Maximum (LGM, Fig. 1a). Immediately south of the PFZ this pattern is reversed: Holocene values exceed glacial values (Fig. 1b). To eliminate the effect of dilution by other phases, such as biogenic carbonate north of the PFZ and detrital sediments south of the PFZ, we estimated opal accumulation rates. In addition, to eliminate the problem of mis-identification of the stage 2/3 and 3/4 boundaries, which might lead to estimating sedimentation rates incorrectly for the stage 2 peak glaciation, we integrated accumulation rates across stages 2–4. This procedure underestimates the true differences in opal accumulation between glacial and Holocene periods as the interstadial stage 3 has opal contents and sedimentation rates intermediate between full glacial and full interglacial stages.

South of 50° S, opal accumulation rates are between two and six times higher during the Holocene than during the last glacial. North of 50° S, this pattern is reversed: opal accumulation is higher during the glacial as far north as 40° S. The pattern north of 50° S is probably the result of a northward shift in the siliceous-ooze belt during the last glacial^{17,22}. However, glacial-to-Holocene changes in integrated opal accumulation rates in the South Atlantic (Fig. 2) suggest that the decrease in opal burial south of the PFZ more than compensated for the increase north of the PFZ, especially as the area south of 55° S almost certainly displayed lower glacial than Holocene opal accumulation (ref. 13 and unpublished data). If opal accumulation patterns in the Indian and Pacific sectors parallel those in the Atlantic (refs 17 and 22), then the integrated opal burial in the entire Southern Ocean south of 40° S was probably reduced during the last glacial.

Diatom species abundances also imply reduced productivity during the LGM south of the present-day PFZ. During the LGM, open ocean forms (dominated by *Nitzschia kerguelensis*) were displaced to the north and replaced to the south by an assemblage dominated by species related to sea ice (*Eucampia antarctica*). The decrease in abundance of open ocean forms during the LGM presumably reflects reduced diatom productivity, a consequence of increased sea-ice cover^{22–24}.

The (Ge/Si)_{Opal} ratio of diatom shells may provide a quantitative record of nutrient uptake. Diatoms incorporate inorganic germanium from seawater into their siliceous shells as if germanium were a heavy stable isotope of silicon^{25,26}. The (Ge/Si)_{Opal} of diatom shells which fall into the deep sea to be

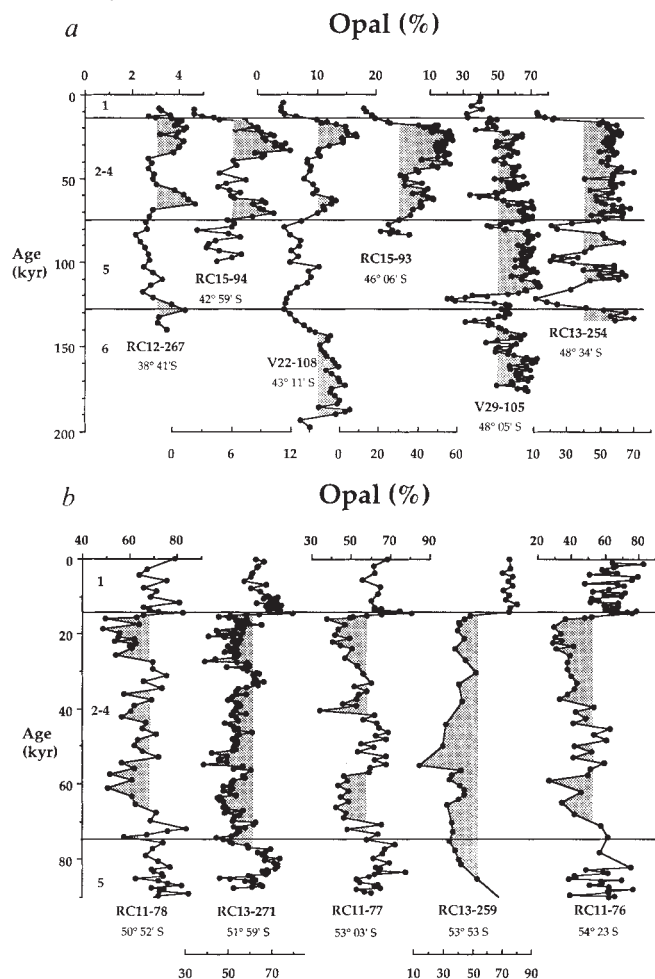


FIG. 1 %Opal against age for cores north (a) and south (b) of the PFZ in Table 1. The cores are displayed north to south from left to right. Shaded regions indicate high and low opal in each core. Numbers indicate isotope stages.

TABLE 1 Piston cores in the Atlantic Sector of the Antarctic Ocean

Core	Latitude	Longitude	Water depth (m)	% Opal†		OAR‡	
				Holo- cene	Glacial	Holo- cene	Glacial
North of the present polar front zone							
RC12-267*	38°41' S	25°47' W	4,144	3	4	0.1	0.1
RC15-94*	42°59' S	20°51' W	3,762	2	8	0.1	0.3
V22-108*	43°11' S	03°15' W	4,171	4	12	0.2	0.3
RC15-93*	46°06' S	13°13' W	2,714	18	46	0.5	3.2
V29-105	48°05' S	17°41' E	4,350	38	57	1.0	2.3
RC13-254*	48°34' S	05°34' E	3,636	16	55	0.5	2.9
South of the present polar front zone							
RC11-78	50°52' S	09°52' W	3,115	72	63	3.0	2.1
RC13-271*	51°59' S	04°31' E	3,634	69	55	13.1	5.6
RC11-77	53°03' S	16°27' W	4,098	65	53	4.4	2.3
RC13-259*	53°53' S	04°56' W	2,677	74	38	2.5	0.9
RC11-76	54°23' S	22°08' W	5,229	65	42	8.5	1.5

* Cores with planktonic and/or benthic $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ records^{16,17}.

† Average %Opal for Holocene and glacial (stages 2–4) sediments.

‡ Integrated opal accumulation rates for Holocene and glacial (stages 2–4) sediments ($\text{g opal cm}^{-2} \text{ kyr}^{-1}$).

buried is fixed by three factors: the Ge/Si ratio of upwelled seawater ($\text{Ge/Si} = 0.735 \times 10^{-6}$), the apparent 'isotopic' fractionation factor, and the fraction of upwelled silica removed from solution into diatoms (Rayleigh distillation)²⁷. Assuming that oceanic (Ge/Si)_{seawater} remained about constant over the last glacial cycle and taking a fractionation factor of ~ 0.5 , Froelich *et al.*²⁸ interpreted an increase in diatom (Ge/Si)_{Opal} at the close of the last glacial in Antarctic core E17-9 (Pacific sector) to reflect increased biosiliceous productivity and decreased preformed silica concentrations. As the (Ge/Si)_{Opal} ratio of glacial-age diatoms was lower than (Ge/Si)_{seawater}, they concluded that glacial Pacific diatoms were less efficient in extracting silica from surface waters. New data from piston cores in the South Atlantic are consistent with the record in Pacific core E17-9 (here extended to Stage 5e) (Fig. 3a). (Ge/Si)_{Opal} of glacial diatoms (Stages 2, 6, 8, 10 and 12: (Ge/Si)_{Opal} = 0.45 to 0.60) are lower than Holocene and interglacial values (Stages 5e, 7, 9 and 11: (Ge/Si)_{Opal} > 0.70). In addition, low (high) (Ge/Si)_{Opal} values coincide with low (high) %opal. The similarities in the short Atlantic and Pacific Ge/Si records, and in the longer Atlantic (Ge/Si)_{Opal} and %opal record of RC13-259 (Fig. 3), suggest that both tracers respond to rapid changes in preformed silica concentrations and biosiliceous production in the Southern Ocean that are roughly synchronous with late Pleistocene climate changes^{18,21}.

We have assumed a simple first-order explanation of these data, that variations in opal accumulation and (Ge/Si)_{Opal} are caused by changes only in local surface-water biosiliceous productivity. Other factors, such as changes in the external silica supply to the ocean (rivers), or changes in deep-sea opal dissolution^{17,29,30}, must be considered. Changes in riverine input of silica and germanium or (Ge/Si)_{rivers} could not produce the rapid (<10,000 yr) shifts in opal burial and (Ge/Si)_{Opal} by whole-ocean changes in (Ge/Si)_{seawater} because the response time of oceanic silica and germanium to external modulations is $\sim 20,000 \text{ yr}$ ²⁸. It is also unlikely that changes in opal dissolution in marine sediments could completely invert the productivity signal, as opal preservation is enhanced by an increased rain of diatoms (higher production) and/or increased bulk sediment accumulation, processes that tend to magnify the sedimentary record of changes in biosiliceous production³¹. Nor is it likely that opal preservation varies greatly as a function of water depth or bottom water chemistry, as deep waters everywhere in the ocean are about equally undersaturated with respect to opal, a factor that is not likely to have changed greatly during the Late Pleistocene. However, if opal dissolution in the deep sea were found to be incongruent with respect to Ge/Si, or if diatom species susceptible to differential dissolution carry different (Ge/Si)_{Opal} ratios, then our (Ge/Si)_{Opal} record could be an artefact of dissolution.

We therefore believe, but cannot yet confirm, that these opal accumulation rates and (Ge/Si)_{Opal} patterns reflect changes in biosiliceous productivity. As diatoms today dominate the export flux of organic carbon from surface waters of the Antarctic^{32,33}, changes in the burial rate of their shells may reflect parallel changes in new primary production, although there is not yet sufficient information to reconstruct palaeosiliceous production from opal burial rates alone.

These data support widely cited but controversial evidence for reduced Antarctic productivity during glacials. The $\delta^{13}\text{C}$ records of Antarctic planktonic foraminifera (*N. pachyderma*) were lower during the last glacial by $\sim 1\%$ ^{12,13,34}. Ignoring the possible effects of gas exchange³⁴ (which can alter $\delta^{13}\text{C}$ independently of nutrients) and a possible global $\delta^{13}\text{C}$ reservoir shift ($\sim 0.3\%$), the lower glacial $\delta^{13}\text{C}$ values imply a reduction in the ratio of biological production to upwelling with higher ΣCO_2 and preformed nutrients at the surface. This implication is now supported by the parallel changes in our $\delta^{13}\text{C}$ (foraminifera), Ge/Si (diatom) and %opal records in RC13-259. In addition, the ratio in the inferred glacial nutrient concentrations [$\delta^{13}\text{C}/(\text{Ge/Si})_{\text{Opal}}$] approximates the present deep ocean P/Si ratio (Fig. 3). Although based on totally different chemical systematics, both records point to higher glacial nutrient and ΣCO_2 concentrations which subsequently fell as productivity increased at the end of the glacial periods.

This simple nutrient-based interpretation of the Antarctic $\delta^{13}\text{C}$ (and Ge/Si) records poses a geochemical dilemma for understanding ocean carbon models and atmospheric CO_2 changes. Broecker and Peng¹⁰ argue that atmospheric CO_2 is controlled by equilibrium with $p\text{CO}_2$ of polar surface waters. With this constraint, the rapid 80 p.p.m.v. increase in atmospheric CO_2 at the close of the glacial periods requires a rapid and dramatic increase in the ratio of ΣCO_2 to alkalinity in Antarctic surface waters. A decrease in ΣCO_2 at the close of the glacials (as our data suggest) requires either a much larger drop in alkalinity than can be reasonably modelled, or else a $p\text{CO}_2$ of Antarctic

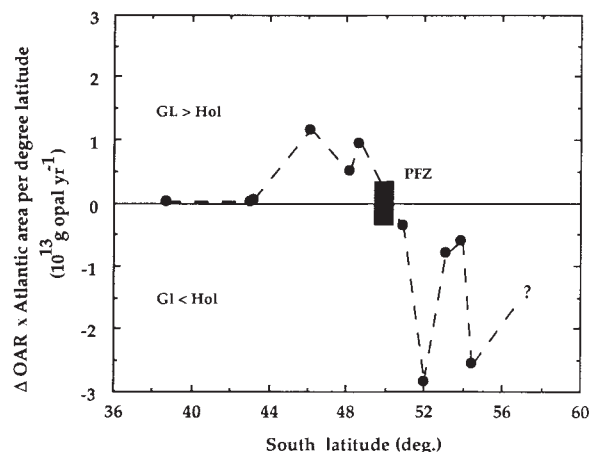
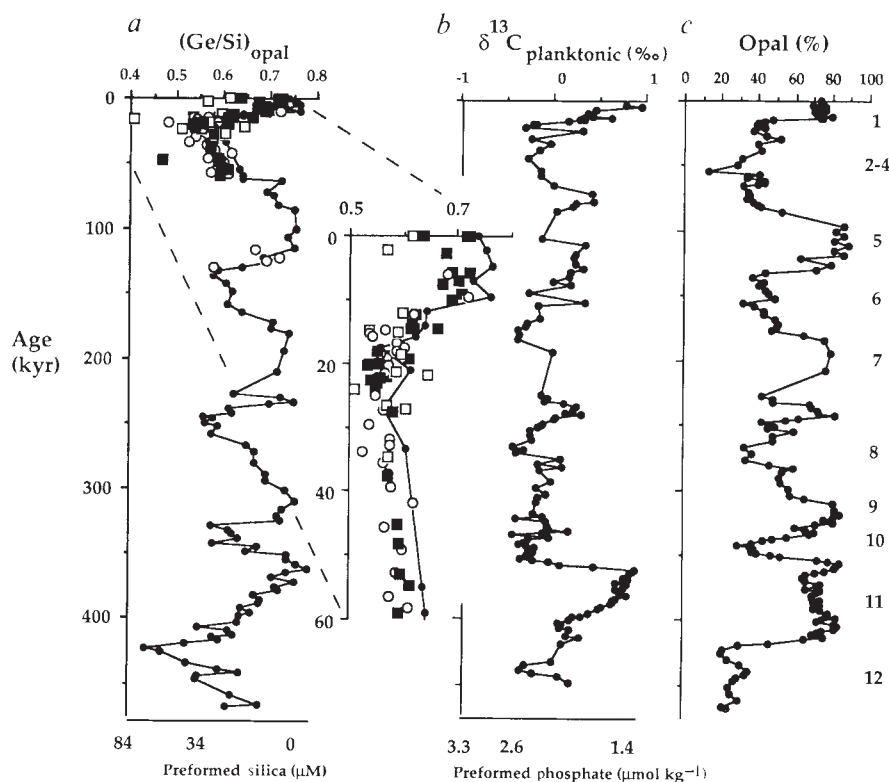


FIG. 2 The glacial-to-interglacial change in opal accumulation in South Atlantic sediments. Opal accumulation rates (OAR) for each core were calculated from: $\text{OAR} = (S/B_0)(\% \text{Opal}/100)$ where S = sedimentation rate (cm kyr^{-1}) and B_0 = dry bulk sediment density (g cm^{-3}) calculated from Froelich *et al.*³⁶. OAR was then integrated over glacial (stages 2–4) and Holocene (Stage 1) intervals of each core, and the Holocene values were subtracted from glacial values to produce ΔOAR — the change (glacial-to-interglacial) in OAR at each core site. ΔOAR was multiplied by the circumpolar area (Atlantic sector: 30° W to 20° E) bounded by one-degree parallels centred on the latitude of each core, and the result plotted against latitude. PFZ marks the position of the present polar front zone. The increase in opal burial north of the PFZ (integrated from 38° to 49° S) is $5 \times 10^{13} \text{ g yr}^{-1}$. The decrease south of the PFZ (50° to 55° S) is $-7 \times 10^{13} \text{ g yr}^{-1}$. Note that a shift in opal burial of $5 \times 10^{13} \text{ g yr}^{-1}$ represents about one-third of the opal burial flux in the South Atlantic and about 10% of the global opal burial flux ($4.5 \times 10^{14} \text{ g yr}^{-1}$)²⁸, almost all of which accumulates south of 40° S ³⁹.

FIG. 3 *a*, (Ge/Si)_{OPAL} (10^{-6} atom per atom) against age in South Atlantic core RC13-259 (solid dots), in the other four Atlantic cores south of the PFZ (solid squares: Table 1), in five Atlantic cores north of the PFZ (open squares: Table 1, all cores except RC15-94) and in South Pacific core E17-9 (open circles). The data can also be read from the preformed surface-ocean silica concentration scale (at bottom), adapted from Froelich *et al.*²⁸. Inset is an expanded record. *b*, $\delta^{13}\text{C}$ (‰) of *N. pachyderma* against age in piston core RC13-259. The data can also be read from the preformed surface-ocean phosphate concentration scale (at bottom), which was adapted from the relation of bulk oceanic $\delta^{13}\text{C}$ with phosphate³¹, ignoring possible gas-exchange effects and the global $\delta^{13}\text{C}$ shift. *c*, %Opal against age in piston core RC13-259.



surface waters that could not have been in atmospheric equilibrium and did not control atmospheric CO_2 . For these reasons, Broecker and Peng¹⁰ suggest that the surface-water $\delta^{13}\text{C}$ signal must be decoupled from nutrients by gas exchange effects and favour Cd/Ca measurements of *N. pachyderma* in Southern Ocean cores, which suggest little or no change in surface-water phosphate or ΣCO_2 ^{11,35}. Until there is more evidence confirming that $\delta^{13}\text{C}$ and Ge/Si, or Cd/Ca, are reliable tracers of nutrient concentrations in Southern Ocean surface-water, we favour the opal accumulation rate data that indicate lower glacial Antarctic production, and suggest that the $p\text{CO}_2$ dilemma results from model-derived rather than from direct evidence.

There is evidence indicating higher non-siliceous production in the glacial Antarctic. Higher methanesulphonic acid (MSA) concentrations and ratios of MSA to non-sea-salt sulphate (n.s.s. SO_4^{2-}) in glacial-age Antarctic ice³⁶ could result from increased Antarctic blooms⁹ of the dimethylsulphide (DMS)-producing prymnesiophyte *Phaeocystis pouchetti*³⁷. But higher glacial MSA/n.s.s. SO_4 could also result from higher DMS emissions from low- to mid-latitude non-siliceous ocean production (coccolithophoridae) and glacial changes in the complex oxidation and transport pathways to the South Pole³⁶. *Phaeocystis* produces no shell to be found in sediments (a *Phaeocystis* biomarker might ultimately provide a palaeoindicator of its production), nor is there evidence that blooms of *Phaeocystis* increase carbon export (as do diatoms), so the MSA evidence from ice cores is more difficult to extrapolate directly to carbon and nutrient changes in Antarctic surface waters.

We suggest that models and mechanisms of atmospheric CO_2 and climate change linked to increased glacial production in the Antarctic are not supported by palaeotracer data from sediments in the Southern Ocean. Glacial biosiliceous productivity may indeed have been lower and upwelled nutrients may have been less efficiently utilized, so that the 'biological pump' did not operate more efficiently in the Southern Ocean during the last ice age. Consequently, biosiliceous productivity may have

been insensitive to a hypothesized increase in iron supply and not directly responsible for the significant glacial drawdown of atmospheric CO_2 (the Antarctic Iron Hypothesis⁹). □

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Reversal records in marine marls and delayed acquisition of remanent magnetization

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RECORDS of geomagnetic reversals 'frozen' into the magnetic components of sediments provide a means to study the time-dependent behaviour of the geomagnetic field during polarity transitions. Although sedimentary records have the advantage of being readily available and continuous, the process by which they acquire a remanent magnetization is still not fully understood¹. Magnetites, which lose their magnetization at relatively high temperatures ($\leq 580^\circ\text{C}$), are generally considered to carry the primary remanence of the sediment—that is, to record the palaeomagnetic direction. Here we describe two reversed-to-normal transitional records from marine marls in which this high-temperature component shows a delayed remanence acquisition relative to a lower-temperature component. In these samples, therefore, the high-temperature component does not reflect geomagnetic changes during the reversal. At least for marine marls, detailed palaeomagnetic, rock-magnetic and geochemical studies are apparently necessary to judge the validity of reversal records.

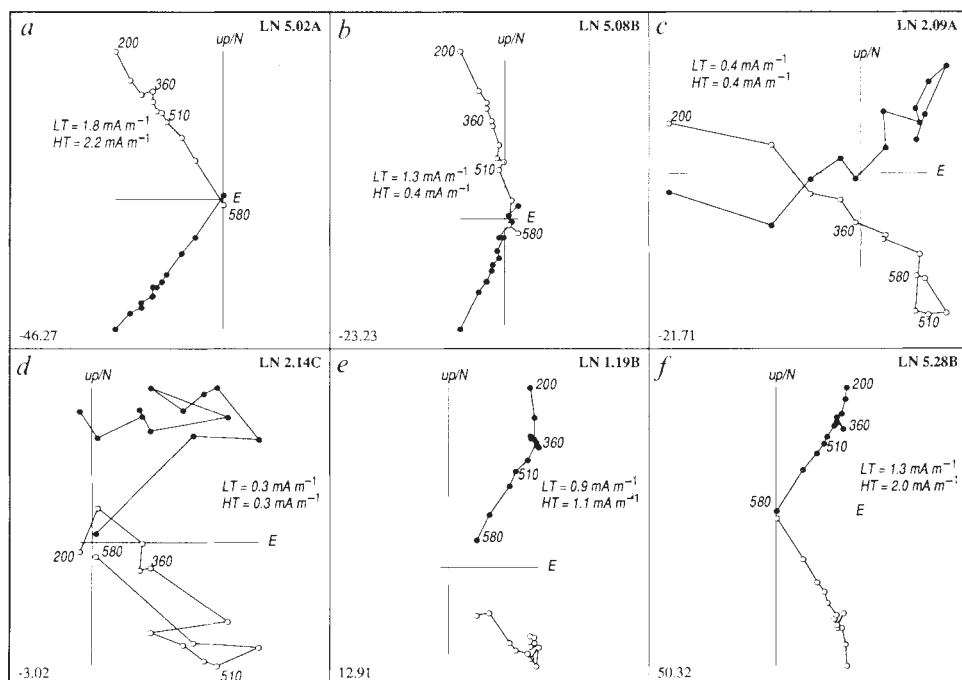
The two studied (R–N) reversals are the lower Nunivak and the lower Cochiti, and are from a succession of 14 reversals which were identified by detailed magnetostratigraphic studies of marine marls on the southern coast of Sicily². These marls are exposed as sedimentary cycles³, each of which shows a characteristic colour layering (see Fig. 2 legend). The sedimentation rate is $\sim 5\text{ cm kyr}^{-1}$ (ref. 4).

The two reversals have been sampled in a cliff section near Eraclea Minoa⁵. Removal of the weathered surface exposes the (blue coloured) fresh marls. Oriented cores were taken more or less parallel to the bedding plane, with an accurately determined stratigraphic spacing. The spatial resolution is a few millimetres, corresponding to a temporal resolution of $<100\text{ yr}$. But the specimen diameter (25 mm) will smooth the remanent magnetization over at least 500 yr.

Typical thermal demagnetizations are characterized by a viscous and/or secondary component removed below 200°C , a low-temperature (LT) component removed between 200 and 480°C , and a high-temperature (HT) component carried by single-domain magnetite⁶ and which is removed at 580°C . Occasionally, slightly higher maximum blocking temperatures are found which indicate the presence of cation-deficient magnetite^{7,8} (Fig. 1c, e).

The LT component decays most strongly between 200 and $330\text{--}360^\circ\text{C}$. Often, there is almost no decay between 360 and 480°C , but some scatter may be observed which is possibly due to magnetic minerals being formed during heating. The HT component decays most strongly between 540 and 580°C . This results in a distinctive and narrow peak in the blocking temperature spectrum which is consistently found not only in the Pliocene marls of Sicily^{2,5} and Calabria^{8,9}, but also in late Miocene marine marls elsewhere in the Mediterranean^{10,11}. The typical HT and LT components recognized in these marls have essentially the same direction, including an inclination error; in southern Sicily these directions also include an average 35° tectonic rotation. In addition, the observed polarity patterns result in an excellent correlation with the geomagnetic polarity timescale². Apparently, both components have been acquired during or shortly after deposition of the sediment. A slightly

FIG. 1 Representative thermal demagnetization diagrams of the lower Nunivak (LN) reversal. Stratigraphic level in cm (left-hand axis) refers to the stratigraphic column of Fig. 2. Solid (open) symbols are horizontal (vertical) projections. Temperature steps below 200°C are not shown in order to enhance the details at seen higher temperatures. Intensities of LT and HT are given in each diagram. Temperature steps are 200, 250, 300, 330, 360, 390, 420, 450, 480, 510, 540, 560, 580°C . a, the HT and LT components are both clearly reversed and include the 35° rotation; b shows the same, except that at the highest temperatures ($>540^\circ\text{C}$) there is a tendency to normal directions and the intensity of the HT component decreases significantly; c, the HT component has a normal polarity, whereas the LT component is still clearly reversed—both components are low intensity; d, the HT component is normal, and the LT component shows an intermediate direction: W and up; e, the LT component has a north direction, but the inclination is still very shallow; f, both components have approximately the same normal polarity direction, including the familiar 35° rotation, and the intensities have largely recovered to pre-reversal values.



delayed acquisition of normal remanent magnetization (NRM)—having no serious effect on the magnetostratigraphic record—may significantly disturb a record of rapid geomagnetic field changes such as occur during a polarity reversal.

Demagnetization diagrams of the different stadia throughout the lower Nunivak transition (Fig. 1) show that the two components were acquired at different times (Fig. 2). The HT component is stably reversed until -23 cm, then there is an abrupt change to normal polarity taking place within 1–2 cm (for explanation of how levels were assigned, see Fig. 2). The transition to normal polarity of the LT component is located ~ 35 cm above the HT reversal and is more gradual. (Note also that the LT component shows normal declinations near -15 cm, in the upper part of the lower white layer.)

A stable HT component residing in magnetite is generally taken to be primary. In a record where the HT component is reversed and the LT component is normal, the latter is easily interpreted to represent a (partial) overprint by secondary (sub-recent) remanences. But if the LT component is reversed and the HT component is normal, as observed in the lower Nunivak reversal record, then the LT component is not a secondary overprint. Apparently, (just) after the R–N geomagnetic field transition has occurred, the HT component records, at a certain depth below the sediment/water interface, the existing (N) geomagnetic field, whereas at that depth the LT component has already locked into the previously existing (R) geomagnetic field. Thus, there is a lag in magnetization between the two components. In the lower Nunivak record, this lag is ~ 35 cm, corresponding to ~ 7 kyr.

There appears to be a relation between lithology and (timing of) acquisition of the two components. This is illustrated by the results of the next R–N reversal, the lower Cochiti (Fig. 3). In the upper part of the record, the grey layer shows a simultaneous reversal of both LT and HT components at a stratigraphic level of 85 cm, implying no time lag. In the beige layer, between levels 35 cm and 25 cm, the HT component is normal and the LT component has already locked the prereversal (R) geomagnetic field indicating a lag of 10 cm. From 25 cm down to 15 cm the

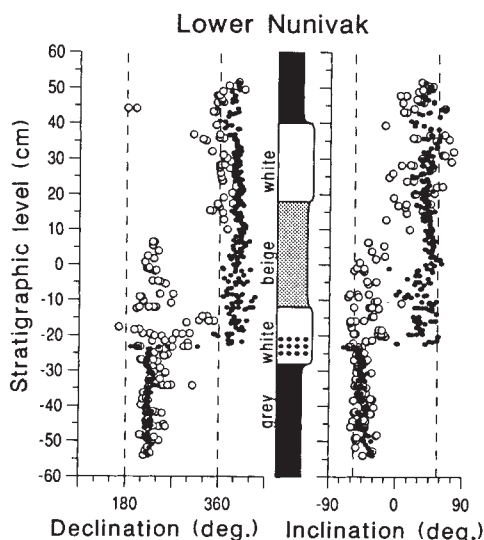


FIG. 2 Declinations and inclinations during the lower Nunivak reversal. Open (closed) symbols denote LT (HT) directions. The LT component reverses polarity ~ 35 cm higher than the HT component and shows more scatter. The weathering colours (carbonate contents⁴) of each sedimentary cycle are grey (70%), white (80%), beige (60%) and white (80%), whereas fresh colours show more gradual changes from dark blue to light blue. Dots in the lower white layer refer to brown oxidation spots. The 0-cm level was (arbitrarily) put at a clearly recognizable (sedimentary) level.

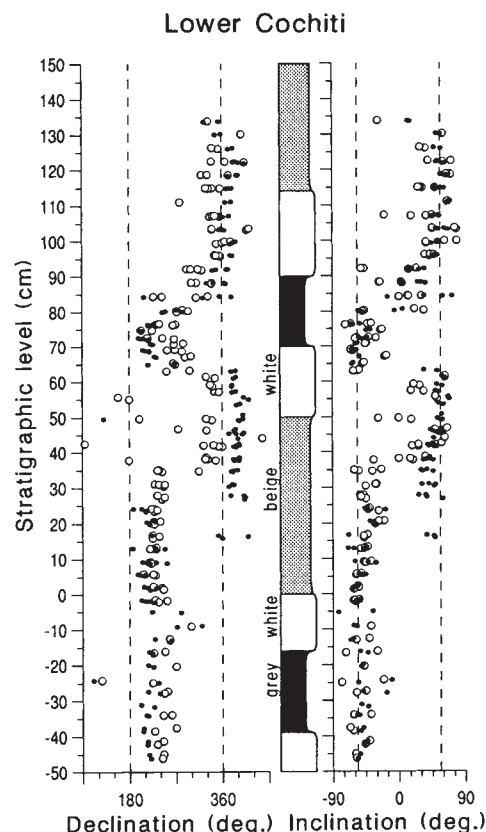


FIG. 3 Declination and inclinations during the lower Cochiti reversal. See also Fig. 2 legend. The record shows three 'polarity reversals', respectively R–N, N–R and N–R, in both the HT and LT directions.

results are ambiguous: the HT component has been interpreted as reversed, but normal polarities are already found at the highest temperatures (similar to the lower Nunivak example of Fig. 1b). The lag may therefore be slightly larger, by as much as 10 cm. Thus, it appears that the HT component lags the LT component of 35 cm (lower Nunivak) and 10–20 cm (lower Cochiti) in the beige and white layers, where in the grey layer there is no apparent lag.

The lower Cochiti record (Fig. 3) could be interpreted as a reversal with a complex geomagnetic field behaviour: either as a R–N transition followed by a R 'rebound' or as a R–N transition preceded by a N 'excursion'. But we find it difficult to believe that the normal polarities in the beige and white layer between levels 35 and 65 cm are due to geomagnetic field changes, whereas those between levels 25 and 35 cm are clearly due to a lag in magnetization. We therefore conclude that the normal magnetizations of both the HT component (between levels 15–25 and 65 cm) and the LT component (between levels 35 and 65 cm) are caused by a delayed NRM acquisition. The absolute timing of the delay is difficult to assess, because the actual reversal may take place anywhere upwards of the level where both components have consistently the same polarity (the 10-cm level in the lower Nunivak; the 95-cm level in the lower Cochiti). The stratigraphic distance between this level and the lowest level that shows a delayed acquisition, provides a minimum estimate of the absolute depth of the lag. In the lower Cochiti for instance, this amounts to an absolute lag of at least 70–80 (60) cm for the HT (LT) component, corresponding to a delay of 14–16 (12) kyr.

The origin of the LT and HT components is likely to be related to physical and chemical processes below the sediment/water

interface, resulting in diagenetic alteration and authigenic formation of magnetic minerals¹. The HT component is carried by fine-grained single-domain magnetite⁶ which may be of biogenic origin (see refs 12–14). Indications of biogenic magnetite were found in late Miocene marls from Crete¹² which show exactly the same demagnetization characteristics¹¹ as the marls studied here. The sharp decay of the HT component between 540 and 580 °C and the corresponding narrow peak in the blocking temperature spectrum may be characteristic of ultra-fine-grained single-domain magnetite in a narrow grain size and possibly of biogenic magnetite. The origin of the LT component is uncertain. The strong decay of this component between 200 and 330–360 °C may indicate that either pyrrhotite or greigite may be a carrier of the remanence. Indications of pyrrhotite have been found in similar marine marls from Calabria⁸. Microbially mediated sulphate reduction to pyrite below the iron-reduction interval in suboxic (to anoxic) sediments may have produced intermediate authigenic forms like greigite^{15,16}. On the other hand, the observed decay between 200 and 330–360 °C may also be an indication of maghemite¹⁷, which may arise from (syn-sedimentary) low-temperature oxidation of magnetite^{18,19,7} under oxic conditions. Rock-magnetic and geochemical studies in our laboratory are presently aimed at determining the magnetomineralogy and palaeoredox indicators.

Marine sediments have been used extensively for detailed geomagnetic reversal studies, many of which are from Mediterranean marls^{8,10,20} having demagnetization characteristics very similar to the Sicilian marls. Our study suggests that, considering the still largely unknown mechanism and especially the timing of remanence acquisition, it is premature to derive characteristics of the geodynamo during reversals from such records. In the records considered here, interpretation of the HT component in terms of geomagnetic field behaviour would lead to an extremely rapid R–N reversal, taking place within 200–400 kyr. In the case of the lower Cochiti, the transition would be followed by a 'rebound' to reversed polarities. Geomagnetic rebounds are probably a real feature of the transition field because they are also observed in volcanic records²¹. But we show that rebounds recorded in (suboxic–anoxic) marine marls may well be due to sedimentary artefacts. In addition, although (oxic) deep-sea marine sediments seem to provide more reliable records of the geomagnetic field, the often cyclic nature of their rock-magnetic and geochemical properties^{22–25} warrants a cautionary interpretation of transitional remanence behaviour in these sediments as well. □

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New early Cambrian animal and onychophoran affinities of enigmatic metazoans

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THERE is much interest in the early evolution of metazoans with the restudy of the Middle Cambrian 'soft-bodied' fauna of the Burgess Shale¹. Several other, newly discovered Cambrian 'soft-bodied' faunas² provide a wealth of new data. One of the oldest and best-preserved faunas was discovered in 1984 in Chengjiang in southern China³. This fauna is of early Cambrian age, about late Atdabanian⁴ (~520–530 Myr BP)². We now describe a new 'armoured lobopod' from the Chengjiang fauna. This animal shows close affinity with the enigmatic *Microdictyon*⁵. The conundrum *Hallucigenia*⁶ is reinterpreted as another 'armoured lobopod', as are *Xenusion*⁷ and *Luolishania*⁸. The large plates set in pairs along the trunk are a synapomorphy of this group, which flourished soon after the 'Cambrian explosion'. Soft-part anatomy suggests that the group has affinities with the Burgess Shale 'lobopod' *Aysheaia*⁹. All these marine, Cambrian forms are here grouped with the extant, terrestrial velvet worms in the phylum Onychophora.

The 'soft-bodied' Chengjiang fauna in the early Cambrian Chiongchussu (Qiongzhusi) Formation occurs at several localities around Kunming in Yunnan, People's Republic of China^{10,11}. It is recognized as the most important early Phanerozoic Lagerstätte beside the Burgess Shale Phyllopod bed^{12,13}. Over 3,000 remarkably preserved, 'soft-bodied' specimens collected to date have revealed a diverse fauna, now partly under study in a Chinese–Swedish project.

In 1989, two laterally compressed specimens of a caterpillar-like animal (Fig. 1) were collected. The 5–6-cm-long trunk bears 11 pairs of legs, above which are rounded, outwardly facing plates. These immediately indicate affinity with the Lower Cambrian problematicum *Microdictyon*¹⁴. Plates of this form known from isolated phosphatic material, were recently found pair-wise above the legs of a worm-like Chengjiang animal⁵ (Fig. 3b). Despite this discovery, the affinities of *Microdictyon* have remained enigmatic^{11,12,15–19}. We regard the paired plates, not noted elsewhere in the animal kingdom, as homologous in the two taxa.

The rounded head of the new form is poorly differentiated from the trunk. There are several thin, elongated structures anteroventrally. At least four of these radiate from a central void, giving the impression of papillae surrounding a mouth. Two elongate structures may be antennae. Three distinct annuli separate neighbouring plate- and leg-bearing segments. Each annulus has a vertical row of about four 'tubercles'. Long, tentacle-like papillae protrude dorsally from the trunk and head. Some annuli carry two such papillae, bilaterally placed, possibly indicating two longitudinal rows. Long, finely annulated protrusions extend ventrally from the trunk, two or possibly more per annulus. The distinctly annulated legs carry large, paired and curved, three-dimensionally preserved claws (Fig. 3a). The trunk merges smoothly with the last leg pair, other legs attach at a circular area beneath each plate. A longitudinal dark band centrally in the trunk apparently represents the gut.

The 10 plate pairs gradually increase in size from the anterior to the fifth pair. Each plate is rounded, dorsoventrally elongate, convex outward, with a ridge near its edge, and a surface pattern of fine pits and nodes. The posteriormost plates are more subrectangular. Each plate is drawn out dorsocentrally (Fig. 2) into a broad-based, pointed spine, directed obliquely upwards and out.

FIG. 1 New 'armoured lobopod' from the Lower Cambrian Chiungchussu (Qiongzhusi) Formation, Maotian hill in Chengjiang County, Yunnan, People's Republic of China. Entire specimen, anterior end to right (Nanjing Institute of Geology and Palaeontology, Academia Sinica (NIGPAS), Nanjing, Cat. No 115271a); compare with Fig. 2. The specimen is compressed and slightly longitudinally rotated clockwise from a lateral view. The plates of the left side, and the corresponding legs, are thus preserved in a more dorsal position than those of the right side. The large, smooth oval adjacent to the dorsal side is an *Isocypris* carapace. Light is from the north-west. Scale bar, 10 mm.



The Chengjiang *Microdictyon* plates are three-dimensionally preserved in minute detail, permitting photography under the scanning electron microscope⁵. They appear to be little altered compared with the isolated phosphatic plates. Plates of the new animal show three-dimensional structures (convexity, ridges, rims) but are thin, black, and apparently unmineralized, resembling the claws. Broadly similar, isolated, *Microdictyon*-like plates with a prominent, pointed spine are known from Greenland (J. S. Peel and S. Bengtson, personal communication). Problematic early Cambrian phosphatic fossils called *Mongolodus*^{18,20} resemble the claws, but are smaller.

The Burgess Shale problematicum *Hallucigenia* has been regarded as representing either a separate phylum or the detached part of some larger organism^{12,15,16,21}. A general resemblance to *Microdictyon* was noted recently^{12,19}. In light of the above, *Hallucigenia* can be reinterpreted as an 'armoured lobopod' (Fig. 4a). The structures originally described as legs⁶ are here regarded as spines of paired plates. The plate edges were previously interpreted to indicate muscle attachment lines. The spines protrude from the upper plate surface. This non-marginal position is paralleled in the new Chengjiang form and in spine-bearing *Microdictyon*.

The 'dorsal tentacles' of *Hallucigenia* are reinterpreted as legs. Only a single row is visible. As in similarly oriented *Aysheaia* specimens⁹, this arises from axial rotation of the trunk. In the holotype of *Hallucigenia*, the bases of two rows of plate spines are preserved at different distances from the upper trunk margin. In 'anterior' pairs two and three this difference in transverse position equals 60–70% of the trunk diameter, indicating up to 45° rotation (assuming that spine-bases were originally positioned near the lateral margin of a cylindrical trunk; $\sin 45^\circ$ gives displacement $0.7r$ on each side, which is 70% of the diameter). This causes an overlap of trunk and legs proximally, previously

interpreted as indicating an internal position of their bases. Bases of the opposing row are therefore, if preserved, positioned under the trunk. Rotation decreases posteriorly, where the five or six 'short tentacles' may well represent three bilateral pairs. The highly sclerotized 'bifid snapper-like structure' terminating *Hallucigenia*'s legs correspond to the paired claws in the new Chinese form. *Hallucigenia* has therefore previously been oriented upside down.

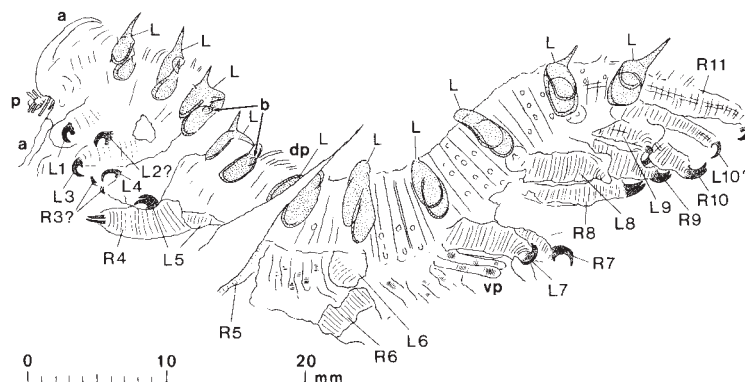
Chengjiang *Microdictyon* support the originally suggested anteroposterior orientation of *Hallucigenia*, although the claws are then anteriorly directed (see below). The inferred posterior trunk in *Microdictyon* (Fig. 3b) is similar to that of *Hallucigenia*. Unfortunately, the 'head' of *Hallucigenia* is unknown. The reconstruction here (Fig. 4a) assumes a head poorly defined from the trunk.

Of debated affinities is also the early Cambrian, Northern European lobopod *Xenusion*^{7,22}. Its trunk and legs are annulated. Dorsolaterally above each leg pair are large, rounded, paired structures. These carry short, central spines, and resist compaction better than the surrounding cuticle. These plate-like structures, combined with general morphology, indicate that *Xenusion* is an 'armoured lobopod'.

*Luolishania*⁸, an annulated lobopod closely similar to *Xenusion*, was recently discovered in the Chengjiang fauna. It has a pair of dome-shaped structures above each leg pair, and a smaller 'tubercle' in between. The 'domes' apparently bear a central process, as in *Xenusion* and some Chengjiang *Microdictyon*. The 'domes' may be homologous with plates, indicating 'armoured lobopod' affinities of *Luolishania*.

The new Chengjiang animal closely resembles the extant, terrestrial velvet worms, constituting the phylum Onychophora. Similarities include the annulated body and lobopod legs with paired claws^{23,24}. Excepting the paired plates, the new animal

FIG. 2 Drawing of new 'armoured lobopod' from Chengjiang, Yunnan, specimen of Fig. 1. The drawing is interpretative, and annulations of legs and ventral protrusions are only symbolically indicated. Left side plates (L) indicated, as are spine bases (b) of two right side plates. Legs of the right (R) and left (L) side are numbered from anterior. R1, R2, and L11 are not exposed; L2 and L10 are tentatively identified. No sediment separates left and right trunk sides, so that annular margins and tubercles of both sides are visible. Two antennae-like structures (a) are tentatively identified. A cluster of papillae (p) may have surrounded the mouth. Some ventral protrusions (vp) are preserved in full length, dorsal papillae (dp) are much thinner and shorter.



could be placed in the Onychophora without much change to the group's morphological concept. This agrees with earlier suggestions of onychophoran affinities of other 'armoured lobopods', including *Xenusion* and *Luolishania*.

The Chengjiang animal is also similar to the Burgess Shale *Aysheaia*⁷, commonly assigned to the Onychophora^{25,26}. *Aysheaia* lacks paired plates, but other structures are closely comparable, indicating affinity. Conversely, the presence of plates allies the new form with *Microdictyon*, *Hallucigenia*, *Xenusion* and *Luolishania*. Accepting the similarities as shared, derived characters, all these forms may now be assigned to the Onychophora.

In both the new Chengjiang animal and *Aysheaia*⁹, the rearward direction of the claws is reversed in the posterior leg pairs. This similarity may indicate either homology and affinity or a similar mode of life. The reversal in claw direction is present also in tardigrades, a group possibly related to onychophorans, and in some crustaceans. In these the character is apparently an adaptation to climbing. Such a lifestyle has been suggested for *Aysheaia*, commonly associated with sponges⁹, and *Luolishania*⁸. Several *Hallucigenia* were found associated with a large worm⁶, and *Microdictyon* from Chengjiang occur in groups on *Eldonia*-like organisms. These associations provide a rare opportunity for inferring behaviour in early forms of life.

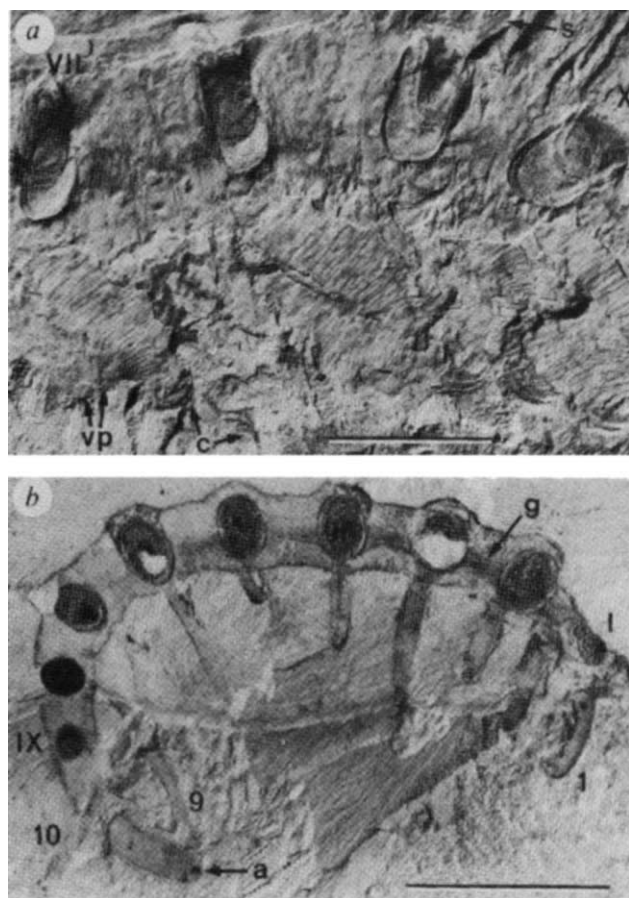


FIG. 3 'Armoured lobopods' from the Chengjiang fauna. *a*, detail of posterior part of new form (specimen of Fig. 1), plate pairs 7 (VII) and 10 (X) numbered. Claws (c), spine (s) of left plate IX, and ventral protrusions (vp) indicated. *b*, *Microdictyon sinicum* (NIGPAS unnumbered). The complete number of legs of one side and plate pairs (I and IX numbered) are preserved, but the anterior(?) termination of the trunk is missing. Legs 1, 9, and 10 (crossing the trunk) are numbered. Full length of legs 1, 2, 4, 9 and 10 preserved. The gut (g) and anus (a) are tentatively identified. Scale bars, 5 mm.

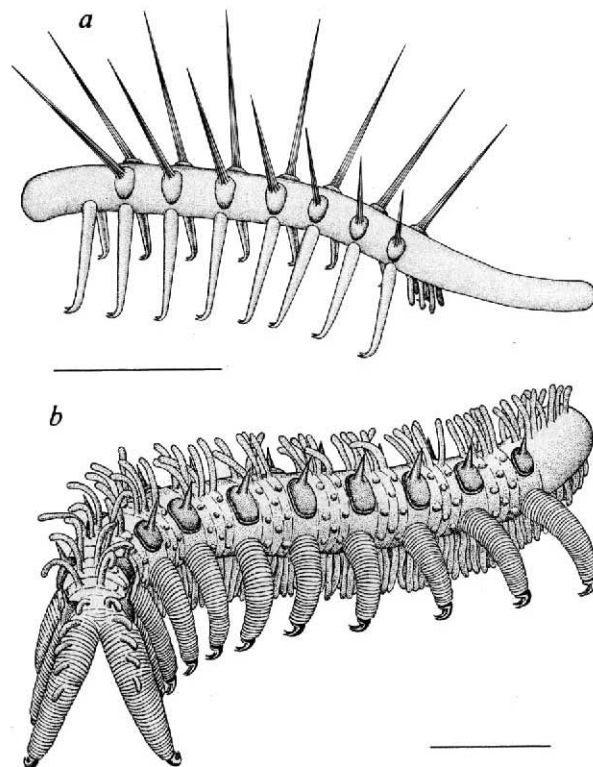


FIG. 4 Reconstructions of 'armoured lobopods'. *a*, *Hallucigenia sparsa*, based on the type material⁶ from the Walcott Quarry near Field, British Columbia. The anterior (?) (facing left) termination is unknown. *b*, new 'armoured lobopod' from Chengjiang, Yunnan, based on specimen of Fig. 1. Head schematic, tentatively identified structures of Fig. 2 not shown, pending further study. Scale bars, 10 mm.

The fauna from Chengjiang and other 'soft-bodied' occurrences provide material for students of early metazoan phylogeny. Two opposite approaches to treating problematica (including *Hallucigenia* and *Aysheaia*), 'shoe-horning'²⁷ versus inferring monotypic phyla^{1,21,28}, were both based on a 'neontological perspective'²⁹ of the evolution of life. They are now challenged by a third alternative, phylogenetic assessments resting on synapomorphies from character analyses²⁹⁻³². □

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Apparent surface curvature affects lightness perception

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THE human visual system has the remarkable capacity to perceive accurately the lightness, or relative reflectance, of surfaces, even though much of the variation in image luminance may be caused by other scene attributes, such as shape and illumination. Most physiological^{1,2}, and computational models^{3–6} of lightness perception invoke early sensory mechanisms that act independently of, or before, the estimation of other scene attributes. In contrast to the modularity of lightness perception assumed in these models are experiments that show that supposedly ‘higher-order’ percepts of planar surface attributes, such as orientation, depth and transparency^{7–10}, can influence perceived lightness. Here we show that perceived surface curvature can also affect perceived lightness. The results of the earlier experiments indicate that perceiving luminance edges as changes in surface attributes other than reflectance can influence lightness. These results suggest that the interpretation of smooth variations in luminance can also affect lightness percepts.

Figure 1b shows a version of the type of illusion used to motivate many models of lightness perception^{3,11,12}. Computational models explain the phenomenal appearance of the front face of the polyhedron using a two-stage mechanism, which we will refer to as edge-integration. A lateral inhibitory process acts on the image to filter out the gradual changes in luminance on the front face, leaving only the step change in the middle. An integration process then fills in constant values of lightness in the two halves, based on the luminance difference across the step change. For more general scenes, the filtering stage factors

out those changes in image luminance caused by smooth variations in incident illumination, leaving the sharp changes due to changes in surface reflectance. What would be seen, however, if the smooth luminance gradients were due not to the illumination of the surface, but to the shape of a smoothly curved surface? Figure 1a shows a modified version of the illusory stimulus in which the bounding contours have been changed from straight to curved. The modified bounding contours, well known cues for surface shape^{7,13,14}, evoke a percept of two abutting cylinders. The effect of such a change in perceived shape on perceived lightness is clear; the difference in perceived lightness of the two halves of the surface disappears when the surface appears curved. The visual system seems to take into account the cause of the smooth luminance gradient before determining surface lightness.

We did a simple experiment to obtain quantitative support for the effect of perceived surface shape on perceived lightness. The experiment made use of a lightness effect first presented by Arend *et al.*¹⁵. They showed that the perceived lightness difference between the two halves of a stimulus like that in Fig. 1b ‘propagates’ to darkened patches placed in the middle of each half of the stimulus. Thus, a patch placed in the phenomenally darker half of the stimulus appears darker than an equally luminous patch placed in the phenomenally lighter half. The difference in perceived lightness of the two patches is consistent with what would be predicted by edge-integration lightness models. If, however, perceived surface shape modulates perceived lightness in the way phenomenally demonstrated in Fig. 1, we would predict the disappearance of the effect for surfaces that appear curved. We tested this prediction by measuring the difference in Arend’s lightness effect for images similar to that of the cylinders in Fig. 1a and images similar to that of the polyhedron in Fig. 1b.

Schematic diagrams of the stimuli used for the experiment are shown in Fig. 2. The strength of Arend’s lightness effect was measured by presenting a darkened patch in one half of a stimulus image and having subjects adjust the luminance of an equivalent patch in the other half of the image so that its apparent surface colour appeared to be the same shade of grey as the test patch (the equal-lightness point). Half of the group of 24 subjects were presented with images of the curved surfaces as stimuli, and the other half were presented with images of the flat surfaces. Because the vertical luminance profiles for the two sets of images were equivalent, the only stimulus variable that differed for the two groups was the form of the bounding contours of the projected surfaces and the target patches. We will refer to the images of the quarter-cylinders as having ‘curved’ boundary contours, and the images of the flat surfaces as having ‘jagged’ contours.

Figure 3 shows the results from the experiment. The 5.8% average increase in luminance needed to make the target patch

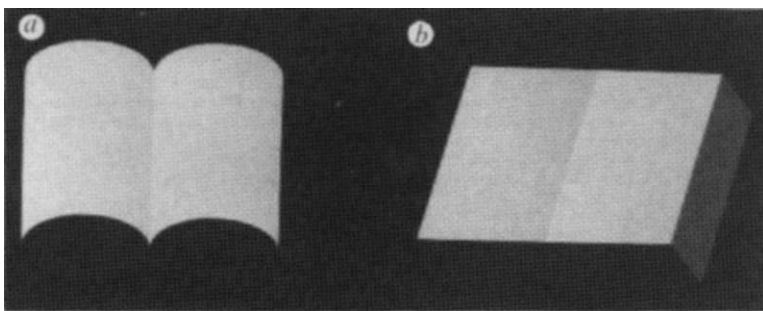


FIG. 1 a, Two abutting cylinders rendered to have a horizontal luminance profile consisting of two linear luminance ramps, shown in c. b, A rectangular polyhedron, the top face of which also has the horizontal luminance profile shown in c. The only difference between the two images is in the shape of

the bounding contours of the surfaces, leading to the different shape percepts, which in turn determine one's percept of the reflectance patterns on the front faces of the two surfaces.

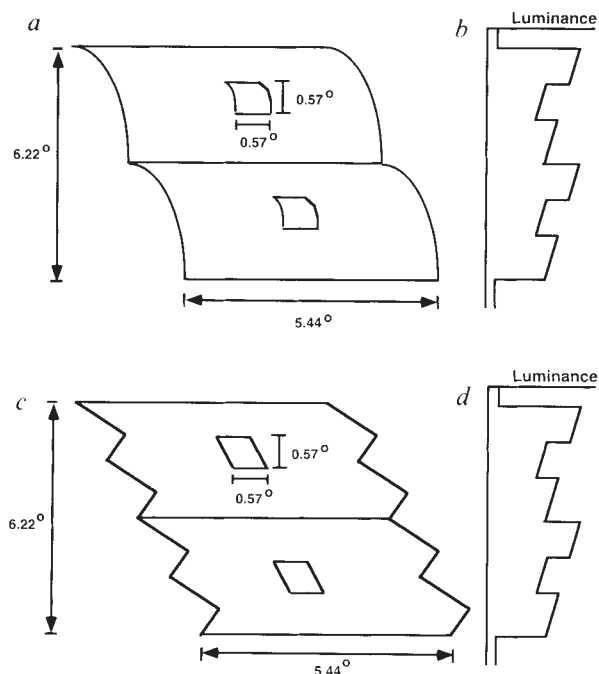


FIG. 2 *a* and *c*, Three-dimensional plot of the surfaces rendered to create the stimulus images. Dimensions are given in visual angle. *b* and *d*, Luminance profile along a vertical cross-section of each stimulus. The parameters defining the shape of the jagged edge boundary were selected to equate its length to the length of the curved edge boundary and to equate the angle made by the boundary at the corner formed in the middle of the stimulus to the angle made by the same corner for the curved edge boundary. Stimulus images were generated on a Stellar GS1000 computer using a three-dimensional rendering program developed by the author. Stimuli were displayed on a Macintosh Ix work-station with an 8-bit colour display monitor. The experiment consisted of a lightness matching task in which subjects were asked to adjust the brightness of a patch in one half of a stimulus image so that its apparent surface colour was the same shade of grey as an equivalent patch in the other half of the image. The half of the stimulus image in which the test patch was placed was varied randomly between trials. In each trial, the luminance of the test patch was scaled by one of three values: 0.7, 0.8 or 0.9. Subjects adjusted the luminance of the matching patch by moving the mouse up or down on a table to vary a factor by which the luminance of the patch was scaled. Subjects indicated a lightness match by pressing the mouse button. A total of 216 trials per subject were used. At the beginning of each trial, the luminance of the matching patch was randomly scaled by a value in the range 0.2–1.8.

in the phenomenally dark half of the images of the flat surface equal in lightness to the patch in the phenomenally light half replicates Arend's result. As predicted by the demonstration in Fig. 1*a*, no significant increase (0.3%) was found for the stimuli with the curved boundary contours. As the two pairs of stimulus images differed only in the form of the boundary contours, we conclude that the main factor underlying the difference in lightness matches was subjects' perception of surface shape.

The results suggest a reanalysis of the phenomena previously considered to support edge-integration models of lightness perception. The visual system does not seem simply to filter out smooth gradients in luminance, but rather takes into account the apparent cause both of smooth and sharp luminance gradients in an image to determine lightness percepts. Either the cooperative interactions in the percepts of lightness and surface shape occur quite early in visual processing, or illusory lightness effects once thought to reflect properties of low-level image processing mechanisms are actually the result of higher-level processes designed to extract information about multiple surface attributes from images. The results support functional

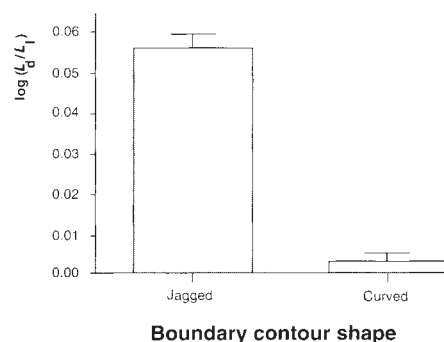


FIG. 3 Results of the matching experiment. In describing the results, we refer to the half of a stimulus image that would appear darker under the illusory lightness effect as the 'dark' half, and the half that would appear phenomenally lighter as the 'light' half. A subject's score on each trial was computed as the log ratio between the luminance of the patch in the 'dark' half of the stimulus image (L_d) and the luminance of the patch in the 'light' half (L_l) at the reported equal-lightness point (regardless of which patch served as the test patch). A score greater than zero indicates that when the patches appeared equally light, the patch in the 'light' half of the stimulus was actually less luminous than the one in the 'dark' half (conversely, such a score suggests that when the patches were equi-luminant, the patch in the light half appeared lighter than the patch in the dark half; Arend's result). The graph shows the average log ratio scores for the two main conditions of the experiment. The average score for the group which viewed stimulus images with jagged surface boundary contours was 0.056, which corresponds to a 5.8% difference in patch luminance at equal apparent lightness. The average log ratio score for the group which viewed stimulus images with curved surface boundary contours was 0.003%, corresponding to a difference of 0.3%. The difference in average scores between the two groups was highly significant ($T(22)=16.8$, $p < 0.0001$).

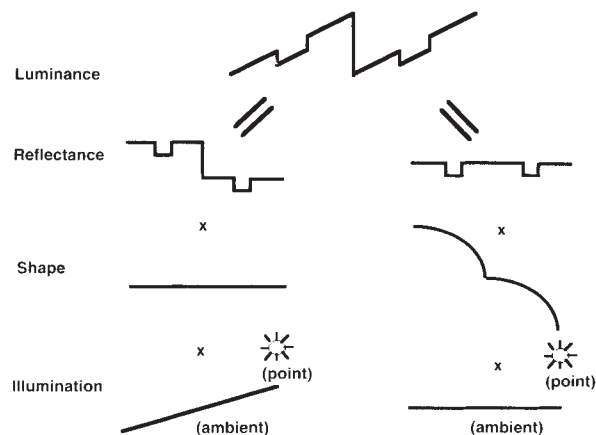


FIG. 4 Two scene interpretations which are consistent with the luminance pattern in the stimulus images used for the experiment. The shape of the surface boundary contour determines the perceived shape of the surface, which determines which of the two interpretations is 'selected' by the visual system.

models of lightness perception that treat lightness as one of a set of attributes comprising a perceptual model of a scene, which the visual system generates cooperatively to maintain consistency between its perceptual model and the luminance data provided in an image¹⁶, as illustrated in Fig. 4. □

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Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor

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ANGIOTENSIN II elicits different responses which affect cardiovascular, neuronal and electrolyte transport regulation¹. To understand the mechanisms responsible for its various actions, the receptor for angiotensin II has long been sought, but numerous attempts to purify the receptor have been unsuccessful owing to its instability and low concentration²⁻⁶. We report here the expression cloning of a complementary DNA encoding a bovine angiotensin II receptor to overcome these difficulties. The receptor cDNA encodes a protein of 359 amino-acid residues with a transmembrane topology similar to that of other G protein-coupled receptors. COS-7 cells transfected with the cDNA expressed specific and high-affinity binding sites for angiotensin II, angiotensin II antagonist and a non-peptide specific antagonist for type-1 receptor. Dithiothreitol inhibited ligand binding. The concentration of intracellular Ca²⁺ and of inositol-1,4,5-trisphosphate increased in the transfected COS-7 cells in response to angiotensin II or angiotensin III, indicating that this receptor is the type-1 receptor for angiotensin II. Northern blot analysis revealed that the messenger RNA for this receptor is expressed in bovine adrenal medulla, cortex and kidney.

We constructed a cDNA library derived from cultured bovine adrenal zona glomerulosa (BAG) cells which had abundant binding sites for angiotensin II, using the mammalian expression vector pCDM8 (ref. 7). A whole library was transfected into COS-7 cells, which had no detectable receptor. The transfected cells were screened for the expression of angiotensin II binding activity by autoradiography using as a probe ¹²⁵I-labelled [Sar¹, Ile⁸]-angiotensin II (sarile), a potent angiotensin II antagonist. We recovered plasmids from COS-7 cells corresponding to positive spots in autoradiograms, and screened repeatedly to isolate a single full-length clone, ARV, which conferred angiotensin II binding capability on COS-7 cells. As ARV lacked part of the 3' non-coding region, we screened the library again by colony hybridization with a 1.2-kilobase (kb)

*Xho*I-*Dra*I fragment of ARV as probe to obtain a full-length clone, ARW.

Figure 1 shows the nucleotide sequence of ARW and its deduced amino-acid sequence. The initiation codon is preceded by an in-frame termination codon and the surrounding nucleotide sequence is in agreement with the Kozak consensus sequence. The polypeptide encoded by the open reading frame consists of 359 amino-acid residues with a relative molecular mass (*M_r*) of 41,093. This *M_r* is in reasonable agreement with that of the deglycosylated form of the angiotensin II receptor (35,000) as determined by SDS-PAGE^{8,9}. Hydropathy analysis

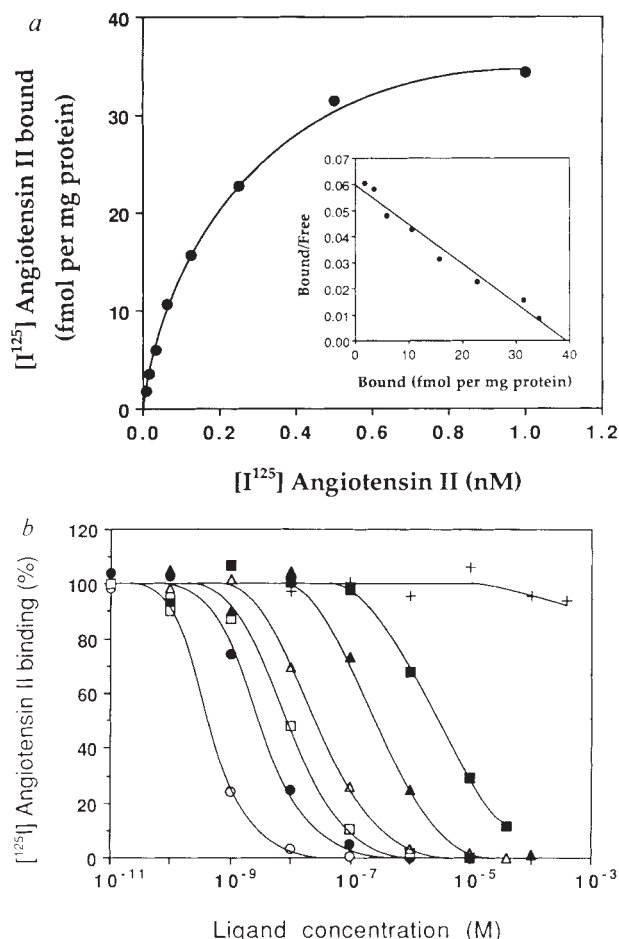


FIG. 2 Binding of ¹²⁵I-labelled angiotensin II to membranes prepared from COS-7 cells transfected with receptor cDNA. *a*, Saturation isotherm of the specific binding of ¹²⁵I labelled angiotensin II to transformed COS-7 cell membranes. Inset shows a Scatchard plot of the same data; *K_d* and *B_{max}* were 0.28 nM and 39 fmol per mg protein, respectively. *b*, Displacement of ¹²⁵I-labelled angiotensin II binding by unlabelled angiotensin I (■), angiotensin II (□), angiotensin III (△), [Sar¹, Ile⁸]-angiotensin II (○), [Sar¹, Ala⁸]-angiotensin II (●), DuP753 (▲) and EXP655 (+). METHODS. A 1.8-kb *Xho*I-*Scal* restriction fragment of ARW was inserted between *Xho*I and *Pst*I (blunt) sites of pCDM8. The resultant construct pAR1.8 was transfected into COS-7 cells by electroporation. Three days after transfection, cells were washed with PBS and scraped off plates. Membranes were prepared according to Zhou *et al.*²⁶ using the homogenizing buffer (100 mM NaHCO₃, 5 mM EDTA, 5 μg ml⁻¹ leupeptin, 5 μg ml⁻¹ pepstatin A, 0.04 mM phenylmethanesulphonylfluoride, pH 8.3) in place of Tris-EDTA-Mg buffer. Binding assays were performed in 250 μl of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 0.2% BSA, 0.06% bacitracin, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ antipain, 10 μg ml⁻¹ chymostatin, 10 μg ml⁻¹ phosphoramidon, ¹²⁵I-labelled angiotensin II (2,000 Ci mmol⁻¹; Amersham) and tested ligand. Binding was initiated by the addition of membranes and carried out at 22 °C for 2 h. Nonspecific binding was determined in the presence of 5 μM unlabelled angiotensin II. ¹²⁵I-labelled angiotensin II (0.1 nM) was used for displacement experiments. Each experiment was performed in duplicate. DuP753 and EXP655 were synthesized for internal use.

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of the amino-acid sequence suggests that seven transmembrane domains are present, with 20–30% sequence identity with those of other G protein-coupled receptors. The protein shows only 20% sequence identity with the human *mas* oncogene product, which had been proposed to be an angiotensin II receptor¹⁹.

The protein contains several potential sites for post-translational modification, which include three consensus sites for *N*-glycosylation¹¹, one of which is in the N terminus preceding the first transmembrane domain and the other two in the third extracellular loop. Several serine and threonine residues are in the second and C-terminal cytoplasmic domains for possible regulatory phosphorylation¹². Other notable features include the hydrophilic N-terminal sequence, the short third cytoplasmic loop, the absence of an aspartate residue in the third transmembrane domain which is implicated in the binding of the charged amine moiety in monoaminergic and muscarinic receptors¹³, and a cysteine residue in the C-terminal region, a possible palmitoylation site^{14,15}. One cysteine residue is present in each of the four extracellular loops; these would probably

form the two disulphide bridges required for ligand binding conformation¹⁶.

To examine the ligand-binding characteristics of this receptor, a 1.8-kb *XhoI-ScaI* fragment of ARW was subcloned into pCDM8 (resulting in construct pAR1.8) and transfected into COS-7 cells. Membranes prepared from these cells bound [¹²⁵I]angiotensin II specifically with a dissociation constant (*K_d*) of 0.28 nM (Fig. 2a), in agreement with the reported value obtained with BAG cells¹⁷. No such binding was detected in untransfected cells or cells transfected with vector DNA. Figure 2b shows competition curves of various ligands with [¹²⁵I]angiotensin II. Dithiothreitol (1 mM) reduced binding by about 50%. Agonists and antagonists competed for the binding of [¹²⁵I]angiotensin II with an order of potency typical of binding to the angiotensin II receptors^{18–20}, with [Sar¹, Ile⁸]-angiotensin II being most potent, followed by [Sar¹, Ala⁸]-angiotensin II, angiotensin II, angiotensin III, Dup753 and angiotensin I. Furthermore, the AT₁-specific antagonist Dup753 (ref. 21) inhibited the binding of [¹²⁵I]angiotensin II completely and the AT₂ receptor-specific antagonist EXP655 (ref. 21) was virtually inactive. Thus, the cDNA that we have isolated encodes a protein with the pharmacological characteristics expected of the AT₁ receptor.

The transfected cells responded to angiotensins II or III with a transient increase in the concentration of intracellular calcium (Fig. 3) and production of inositol phosphates. Angiotensin I at the same concentration (10 nM) had no effect on the rise in [Ca²⁺]_i. The ARW-transfected COS-7 cells responded to angiotensin II (100 nM) with a rapid production of inositol 1,4,5-trisphosphate, with accumulation over 30 min of inositol monophosphate and bisphosphate (data not shown).

A 3.3-kb band corresponding to the receptor mRNA was detected in bovine adrenal cortex, adrenal medulla and renal medulla by northern blot analysis (Fig. 4).

Angiotensin II mediates a variety of responses in different tissues. The application of selective high-affinity non-peptide antagonists have revealed the existence of receptor subtypes. One of the subtypes (AT₁) is sensitive to Dup753 (ref. 21) or EXP6803 (ref. 19), and the other (AT₂) to EXP655 (ref. 21), WL19 or CGP42112A (ref. 22). AT₁ seems to be the predominant angiotensin II receptor coupled to inositol phosphate metabolism and regulates vascular contractility and blood pressure¹⁹. In contrast, no role has yet been ascribed to AT₂ (ref. 21). The selective sensitivity to AT₁-specific non-peptide antagonists, transient calcium and phosphoinositol responses and inhibition of ligand binding by dithiothreitol, all support the conclusion that the present clone encodes an AT₁ (type-1) angiotensin II receptor. □

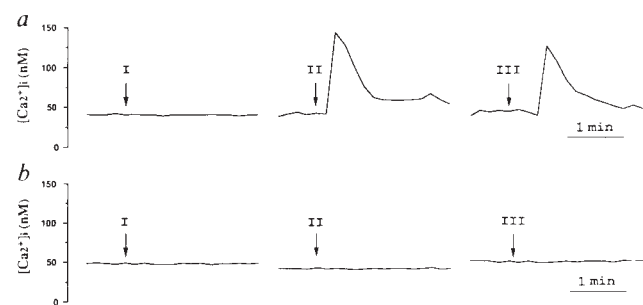


FIG. 3 Changes of [Ca²⁺]_i in COS-7 cells transfected with the receptor cDNA (a) or vector plasmid (b) in response to angiotensins I, II and III. Typical examples of response of single cells are represented.

METHODS. COS-7 cells were transfected with pAR1.8 or vector plasmid pCDM8 and grown on the polylysine-treated coverglass using Flexiperm (TOYOBO). Two to three days later, cells were washed twice with PBS and incubated in PBS containing 5 μM Fura2-AM (Dojin) for 1 h at 37 °C. The Fura2-loaded cells were washed twice and subjected to the analysis. Cell fluorescence was measured with excitation at 340 nm and 380 nm, and emission at 500 nm. Cells were perfused with PBS containing 0.1% BSA and ligand at a flow rate of 0.8 ml min⁻¹.

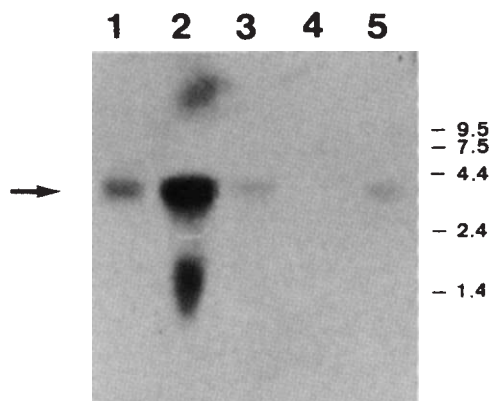


FIG. 4 Northern blot analysis of RNA from bovine tissues. The 3.3-kb receptor mRNA is indicated by an arrow. Each lane contains 10 μg total RNA. Size markers (kb) are shown on the right. Lane 1, BAG cells; 2, adrenal cortex; 3, adrenal medulla; 4, renal cortex; 5, renal medulla.

METHODS. Total RNA was isolated using the guanidine isothiocyanate-CsCl method. Northern hybridization was performed according to the manufacturer's recommendation using Hybond-N + nylon membrane (Amersham) and the 1.2-kb *XhoI-DraI* fragment excised from clone ARW as probe. The membrane was washed in a wash buffer, 0.1 X SSPE (15 mM NaCl, 1 mM NaH₂PO₄, 0.1 mM EDTA, pH 7.4), 0.1% SDS at 50 °C and autoradiographed for 3 h at -80 °C with two intensifying screens.

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Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor

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ANGIOTENSIN II is an important effector molecule controlling blood pressure and volume in the cardiovascular system¹. Its importance is manifested by the efficacy of angiotensin-converting enzyme inhibitors in the treatment of hypertension and congestive heart failure^{2,3}. Angiotensin II interacts with two pharmacologically distinct subtypes of cell-surface receptors, AT₁ and AT₂ (ref. 4). AT₁ receptors seem to mediate the major cardiovascular effects of angiotensin II^{5,6}. Here we report the isolation by expression cloning of a complementary DNA encoding a unique protein with the pharmacological specificity of a vascular AT₁ receptor. Hydrophobic modelling of the deduced protein suggests that it shares the seven-transmembrane-region motif with the G protein-coupled receptor superfamily⁷. Knowledge of the AT₁ receptor primary sequence should now permit structural analysis, definition of the angiotensin II receptor gene family and delineation of the contribution of AT receptors to the genetic component of hypertension.

Mammalian cell expression cloning is a powerful approach to isolate cDNAs encoding cell surface and other proteins^{8,9}. We prepared a plasmid pCDM8 cDNA expression library from rat aortic vascular smooth muscle cells, which express high levels of AT₁ receptors¹⁰. For the first round of screening, 4.3×10^4 clones were amplified in 144 pools of ~300 clones. Single dishes of COS-7 cells transfected with plasmid DNA prepared from each pool were screened within 60 h by binding of 0.2 nM [¹²⁵I]Sar¹, Ile⁸-angiotensin II (sarile) for angiotensin II receptor-like expression. [¹²⁵I]Sarile binding to two pools (Ca18 and Ba23) of transfected cells was 1.5- to 2-fold above the average signal observed in the 24 pools. Pool Ba23 was screened by radioligand binding until clone plasmid pBa23.i401 containing a 3,141-basepair (bp) cDNA insert was isolated. Using a cDNA fragment from this clone as a probe, pool Ca18 was screened by colony hybridization, and a second clone, plasmid pCa18b, containing a 2,213-bp insert was isolated.

Radioligand binding assays in membranes prepared from COS-7 cells transfected with pBa23.i4 indicate that the clone encodes a protein with the pharmacological characteristics of an AT₁ receptor subtype (Fig. 1a). The dissociation constant (K_d) (0.68 nM, $n=2$) from saturation binding studies using [¹²⁵I]sarile is within the range reported for an AT₁ receptor^{6,11–13}. Little or no specific [¹²⁵I]sarile binding is observed in membranes from COS-7 cells transfected with pCDM8 as control (data not shown). The pharmacological profile of ligands competing for [¹²⁵I]sarile binding to the expressed clone is also consistent with that of an AT₁ receptor^{6,11–13} (Fig. 1b; Table 1). The potency series of angiotensin II > angiotensin III > angiotensin I is similar to that for adrenal cortical¹², renal glomerular¹¹ and liver⁶ AT₁ receptors. The affinity of Dup753, an antagonist for AT₁ but not AT₂ receptors, is also within the range reported in other AT₁-expressing tissues^{6,12,13}. The AT₂ receptor-selective

TABLE 1 Competition binding profile of COS-7/Ba23.i401 membranes

Ligand	K_i (nM) $\pm$ s.e.m.	n
Sar ¹ -angiotensin II	0.38 $\pm$ 0.07	3
Sar ¹ , Ala ⁸ -angiotensin II	0.43 $\pm$ 0.08	3
Sar ¹ , Ile ⁸ -angiotensin II	0.46 $\pm$ 0.03	3
Ile ⁷ -angiotensin III	1.1 $\pm$ 0.3	3
Angiotensin II	1.6 $\pm$ 0.2	3
Angiotensin III	5.4 $\pm$ 0.5	3
Dup753	6.3 $\pm$ 0.9	3
Angiotensin I	74 $\pm$ 12	3
Arg ⁸ -vasopressin	>1,000	2
Endothelin	>1,000	2
PD123177	>10,000	2

K_i (nM) values were derived from the relationship $K_i = IC_{50}/1 \pm (F/K_d)$. IC_{50} values were determined using the nonlinear regression, single-site competition curve-fitting function of Graphpad Inplot. F is the concentration in nM of radioligand added in each experiment and K_d is the affinity constant of [¹²⁵I]sarile (0.68 nM) derived from saturation binding assays.

ligand PD123177⁶, which has low affinity for AT₁ receptors, does not compete for radioligand binding in membranes from transfected cells at concentrations up to 0.1 mM. Vasopressin and endothelin-1 do not compete for [¹²⁵I]sarile binding at concentrations up to 1 μ M. Binding of ligands to AT₁ receptors, but not AT₂ receptors, is sensitive to sulphhydryl reducing agents¹⁴. Dithiothreitol inhibits radioligand binding to the expressed clone with a 50% inhibitory concentration (IC_{50}) of 1 mM, where over 95% of radioligand binding is eliminated at a concentration of 10 mM.

A characteristic of the AT₁ receptor in vascular smooth muscle is its ability to activate phosphoinositide-specific phospholipase C (PI-PLC) and to mobilize intracellular stores of Ca^{2+} (ref. 15). Signal transduction was characterized in CHO-K1 cells constitutively transfected with pBa23.i401. Angiotensin II (100 nM, 30 min) increases total inositol phosphate accumulation by $93 \pm 9\%$ above that observed in unstimulated, transfected cells, consistent with coupling to a PI-PLC (Fig. 1c). No formation of inositol phosphates is observed in response to angiotensin II in untransfected CHO-K1 cells. Exposure of transfected cells to 100 nM angiotensin II induces a 1.9-fold transient increase in cytosolic free Ca^{2+} , which rapidly decays to a sustained plateau considerably elevated above the basal level (Fig. 1d). Angiotensin II does not mobilize Ca^{2+} in untransfected cells.

The cDNAs of pBa23.i401 and pCa18b differ only in their untranslated regions (Fig. 2). Each cDNA begins at the same residue; however, an 88-bp sequence found in the 5' untranslated region of pCa18b is not present in the corresponding region of pBa23.i401. In the 3' untranslated region both clones are identical up to nucleotide 1,920 of pBa23.i401, where pCa18b contains five additional nucleotides before ending with a poly(A)⁺ track. Plasmid pBa23.i401 cDNA extends an additional 1,028 bp 3' before ending with a second region of poly(A)⁺. Except for these differences both cDNAs are identical. The implications and functional significance of these untranslated region differences remain to be determined.

An identical 1,077-bp open reading frame in both clones begins at the first in-frame ATG in pBa23.i401 or the first in-frame ATG in pCa18b following a nonsense codon (Fig. 2). This ATG more nearly approximates a consensus translation initiation site¹⁶ than does a second in-frame ATG at nucleotide 88 and is probably the translation initiation codon. The open reading frame encodes a protein of 359 amino acids, and relative molecular mass (M_r) 40,889, sharing structural features with G protein-coupled receptors¹⁷. A search of the NBRF database indicated that this protein had not been previously described. Seven regions of hydrophobic residues corresponding to prospective transmembrane domains are identifiable. The protein

contains three potential asparagine-linked glycosylation sites; one at Asn 4 corresponding to an extracellular N-terminal region and two within a prospective second extracellular loop at Asn 176 and Asn 188. Glycosylation of these residues may be responsible for the difference between the calculated M_r of the deduced protein and that determined in receptor crosslinking studies¹⁷. Cys 101, Cys 180 and Cys 274 are found on extracellular domains I, II and III, respectively. Two of these, Cys 101 and Cys 180, correspond to cysteines found in the first and second extracellular loops of rhodospin that are essential for both receptor processing and sensitivity to reducing agents¹⁸.

An aspartate commonly found in the third transmembrane domain of amine receptors is absent in the AT₁ receptor, as in other peptide hormone receptors^{19–22}. Ser 342 in the C terminus of the receptor corresponds to a consensus cyclic AMP-dependent protein kinase phosphate acceptor²³.

High stringency northern hybridization analysis of polyadenylated RNA using a full length pCa18b probe reveals 2.3 kb and 3.5 kb hybridizing transcripts in vascular smooth muscle and other tissues (Fig. 3). The sizes of the cloned cDNAs are consistent with these transcript sizes. The smaller transcript is the more abundant of the two and is present in all rat tissues

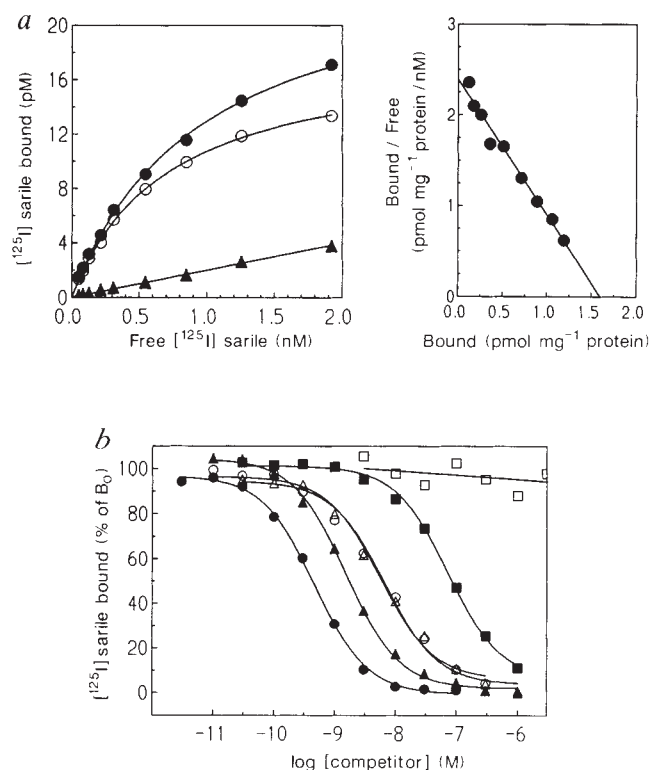


FIG. 1 Pharmacological and functional characterization of cloned AT₁ receptor expressed in mammalian cells. **a**, Representative [¹²⁵I]sarile saturation binding curves in membranes prepared from pBa23.i401-transfected COS-7 cells. ●, Total binding; ▲, nonspecific binding; ○, specific binding; (left panel). Rosenthal transformation of the specific binding data (right panel). **b**, Ligand competition for [¹²⁵I]sarile binding in pBa23.i401-transfected COS-7 membranes. ●, sarile; ▲, angiotensin II; △, angiotensin III; ○, Dup753; ■, angiotensin I; and □, PD123177. Competition data are presented as a percentage of specific binding in the absence of competitor where each point is the mean of three duplicate experiments. **c**, 100 nM angiotensin II-stimulated inositol phosphate accumulation as a function of time in untransfected and pBa23.i401/pSV₂-hygromycin-transfected CHO-K1 cells. **d**, Representative tracings of angiotensin II-stimulated cytosolic free Ca²⁺ transient in untransfected and pBa23.i401/pSV₂-hygromycin-transfected CHO-K1 cells. The arrow indicates the time of angiotensin II application (100 nM).

METHODS. Membrane preparation and binding assays: COS-7 cells grown on 10-cm culture dishes were transfected with 1.0 µg of CsCl-purified plasmid as described in Fig. 2 legend. Crude particulate fractions were prepared as described²⁹ and aliquots were stored at -80 °C until used in binding assays. Binding assays were done in duplicate or triplicate. Nonspecific binding was defined as binding in the presence of 1.0 µM angiotensin II. Membranes (485 µl; 6 µg protein) suspended in 25 mM Tris-HCl, 5 mM MgCl₂, 0.1% BSA were added to tubes with 5 µl inhibitor. The assays were initiated by adding 10 µl of [¹²⁵I]sarile (0.2–0.3 nM final assay concentration for competition assays) (2,175 Ci mmol⁻¹; New England Nuclear) and incubated at 22 °C for 45 min. The suspension was aspirated over GF/B filters using a Brandel cell harvester and washed three times with 4 ml of ice-cold 50 mM Tris-HCl, pH 8.0, and counted on a Beckman gamma counter. Under

these conditions, total binding in the absence of competitor was generally 1–2 × 10⁴ c.p.m. per tube and nonspecific binding was about 500–800 c.p.m. Binding data were analysed using the nonlinear regression curve-fitting routines of Graphpad Inplot. Functional assays: CHO-K1 cells were co-transfected with 20 µg pBa23.i401 and 0.2 µg pSV₂-hygromycin by the precipitation method³⁰. Transfected cells were population-selected for 14 days in a growth medium supplemented with 400 µg ml⁻¹ hygromycin B (Calbiochem). Constitutive expression was monitored by [¹²⁵I]sarile binding (data not shown). The cells were grown in DMEM supplemented with 10% FBS, penicillin/streptomycin and 200 µg ml⁻¹ proline before plating for functional assays. Inositol phosphate measurement: Untransfected and transfected CHO-K1 cells were labelled with 10 µCi ml⁻¹ [³H]myoinositol for 24 h, washed, and incubated for 15 min in a balanced salt solution containing 10 mM LiCl. The cells were exposed to 100 nM angiotensin II or buffer alone for 10, 20 or 30 min in the continued presence of lithium. The reactions were terminated and processed for determination of inositol phosphates as previously described³¹. Total inositol phosphates were eluted from a 0.5 ml Dowex AG-1X8 column with two 5-ml washes of 1 M formate–0.1 M formic acid after thoroughly washing the column with water to remove free inositol. Data are expressed as a per cent (mean ± s.e.m.) of the average control basal value (untransfected controls: 1,783 ± 137 d.p.m.; transfected controls 1,555 ± 128 d.p.m.) for a representative experiment. Cytosolic Ca²⁺ measurements: cells were scraped from 10-cm culture dishes in divalent HBSS (Hank's balanced salt solution containing Ca²⁺ and Mg²⁺), resuspended and incubated for 30 min at 37 °C in balanced salt solution containing 5 µM fura-2/acetoxymethyl ester, washed, and placed on ice until use. To measure fluorescence, cells were resuspended at a concentration of 1 × 10⁶ cells ml⁻¹ and fluorescence was monitored in an SLM/Aminco DMX 1000 spectrofluorimeter at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. [Ca²⁺]_i was calibrated and computed as described⁴⁵. In six experiments on transfected cells, [Ca²⁺]_i increased following the addition of 100 nM angiotensin II from 59 ± 7 to 113 ± 8 nM, and did not increase in untransfected cells (51 ± 10 to 68 ± 15 nM).

examined with the exception of testes and whole brain. The 3.5-kb transcript is in those tissues expressing abundant levels of hybridizing mRNA such as liver, kidney, adrenal and lung and is present in much lower amounts in heart, thymus, uterus, ovary and aorta, where the 2.3-kb hybridization signal is much weaker. This tissue distribution of mRNA is consistent with the

known distribution of angiotensin receptors²⁴, although the AT₁ subtype is best characterized only in adrenal¹², liver⁶ and kidney¹¹. The two transcript sizes may represent either alternative processing of a common RNA precursor, or two highly similar gene products. Additional experiments will be required to test these possibilities.

FIG. 2 Nucleotide and deduced amino-acid sequence of the rat vascular AT₁ receptor clones. The primary nucleotide sequence shown is for the pBa23.i401 cDNA and its deduced amino-acid sequence. Those nucleotides where pCa18b differs from pBa23.i401 are indicated in lower case letters but do not change the numbering system for pBa23.i401. The most 3' sequence of pCa18b is underlined twice. The seven proposed transmembrane domains are underlined once. The Asn-linked glycosylation sites are marked with an asterisk.

METHODS. Poly(A)⁺ RNA was prepared from passage number 12 vascular smooth muscle cells and size-fractionated on a linear sucrose density gradient. cDNA prepared with either random hexamer or oligo(dT) primers were size-fractionated and ligated into the *Bst*X1-compatible sites in pCDM8. A 5 × 10⁵ clone cDNA library with an average insert size of 2.0 kb was constructed in *Escherichia coli* MC1061/p3 by electroporation. The library was amplified on LBAT plates (LB agar with 12.5 µg ml⁻¹ ampicillin and 7.5 µg ml⁻¹ tetracycline) in six sets of 24 pools, 200 to 400 clones per pool. Plasmid DNA was prepared from each pool by the alkaline lysis miniprep method and 1/5 was transfected over 3.5 h using DEAE/dextran into 4 × 10⁵ COS-7 cells cultured on 6-cm dishes in DMEM with 10% Nuserum/5% CO₂. Within 60 h, the dishes were washed with HBSS and incubated for 45 min at room temperature with 0.2 nM [¹²⁵I]sarile in HBSS, 0.1% (w/v) BSA, washed with ice-cold HBSS three times, then dissolved in 0.2 M NaOH and analysed by gamma radiation spectroscopy. For the secondary screen, 24 subpools of 50 clones each were prepared from pool Ba23 and screened as described for the first round. For the final screen, 48 individual clones were picked from the master plate of a positive subpool, grown in liquid culture and plasmid DNA prepared and screened as before. Plasmid pBa23.i401 is the only clone identified in the third screen. To obtain pCa18b, colonies from pool Ca18 were attached to nitrocellulose membranes and hybridized with a pBa23.i401 *Eco*RI-*Pst*I cDNA fragment labelled with [³²P]dCTP by the random primer method and washed at high stringency for 1 h (62 °C, 0.5 × SSC, 0.1% SDS (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0)). *Hind*III-*Not*I fragments from both clones were subcloned into pBluescript KS(±) and sequenced on both strands with Sequenase (US Biochemicals). Secondary structural was analysed using the Wisconsin GCG package on a VAX computer.

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-182 AGGACTGGATGCTGCTCCCGCTGGAGAG -152
GATTCGTGGCTTGGTCTCTCCACCCGATCACCAGTACCCGGGTGGCGAGCCGGT -92
ACCAGCGCTGCGGCTCTCTCAGCTCTGCCACATTCCCTGagtaacatgatgagattgg -52
tataaaatggctgcttctgtctggaataacacacacacccctccagaaagtgtaccac
aggtaaagGTCAAGTGGATTTCGAATAGTGTCTGACACCACTCAACCCAGAAAAACAA -1

1  ATGGCCCTTAACCTCTCTGCTGAAGATGGTACAAAGAATCCAAGTACTGCCCAAG 60
   M A L N * S S A E D G I K R I Q D D C P K
   GCTGGCAGGCACAGTACATATTGTCTATGATCCCTACCTCTACAGCATCATCTTTGT 120
21  A G R H S Y I F V M I P T L V S I I F V
   GTGGGAATATTGGAAACAGCTTGGTGGTGGTTCATTTCATTACATGAAGCTGAAG 180
41  V G I F G N S L V I V I Y F Y M K L K
   ACTGTGGCTGCTCTCTCTCAATCTCGCTCTGGCTTATGCTTTTGTGCTACT 240
61  T V A S V F L L N L A L A L D L C F L L T
   TTGCCCTGTGGCAGTCTATACCGCTATGGAGTACCGCTGGCCCTTCGGCAATCACTA 300
81  L F L W A V Y T A M E Y R W P F G N H L
   TGTAAAGTCTCGGCCAGCTGAGCTTCAACCTCTACGCCAGTCTGCTTCTCTCACG 360
101 C K I T A S A S V S F N L Y A S V F L L T
   TCTCTACAGCATCGACGCTACCTGGCCATCGTCCACCAATGAAGTCTGCTCTCCGCC 420
121 C L S I D R Y L A I V H P M K S R L R R
   ACGATGCTGGTGGCCAAAGTCACTGCATCATCTGCTGCTGATGGCTGTGGCCAGT 480
141 T M L V A K V T C I I I W L M A G L A S
   TTGCCCTGTCTATCCACCAAGTATATCTATCTACGAGAACCACTATCACTAGTGGC 540
161 L P A V I H R N V Y F I E N T * I T V C
   GGGTTTCATTAGTCTCGGAATTCGACGCTCCCATGGCTGGCCCTTACCAAGAA 600
181 A F H Y E S R N * S T L P I G L L T K N
   ATTCGGGCTCTGTCTCTCTCTCTATCTCTACAGCTATACCTCTATCTGGAAA 660
201 I L G F L F P F L I L T S Y T L I W K
   GCTCTAAGAGAGCTTATGAATTCAAAGCAACAAAGCAAGCAAGCAAGCAAGTCTTAG 720
221 A L K K A Y E I Q K N K P R N D D I C A T
   ATAATTGGCGATGTGCTTTCTTCTTCTTCTGCTGGCTCCCGCCACCAATATTCT 780
241 I I M A I V L F P F F S W V P H Q I A T
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261 F L D V L I Q L G V I H D C K I S D I V
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281 D T A M P I T I C I A Y F N N C C L N P L
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301 F Y G F L G K K F K Y F L K L K Y I
   CCCCAGAGGCGCAAGTCCCACTCAAGCTCTGCTACGAAATGAGCAGCGCTTCTTAC 1020
321 P K A K S H S L S T K M S T L S Y R
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341 P S D N M S S S C A K A A A S C F E F #

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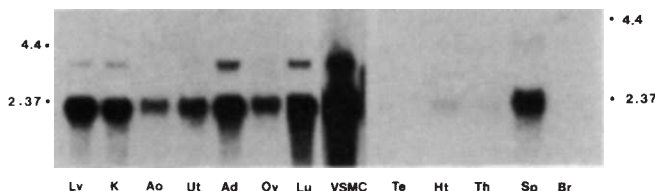


FIG. 3 Northern hybridization analysis of polyadenylated RNA prepared from rat tissues. Lv, liver; K, kidney; Ao, aorta; Ut, uterus; Ad, adrenal; Ov, ovary; Lu, lung; VSMC, vascular smooth muscle cells; Te, testes; Ht, heart; Th, thymus; Sp, spleen; and Br, whole brain. The relative position in kilobases of high-*M_r* RNA marker fragments are indicated.

METHODS. Polyadenylated RNA extracted from tissues was electrophoretically resolved on 1.2% agarose-0.67% formaldehyde gels in a buffer containing 40 mM MOPS (3-[*N*-morpholino]propanesulphonic acid), 10 mM sodium acetate and 1 mM Na₂EDTA. On transfer to nylon membranes (Hybond N, Amersham), the blots were prehybridized for 2 h then hybridized for 16 h with a random primed (T7-polymerase, [³²P]-dCTP-labelled) 2.2-kb *Hind*III-*Not*I double-stranded Ca18b cDNA probe at 42 °C in a buffer containing 1 M NaCl, 50 mM Tris-HCl, 5 × Denhardt's (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 50% formamide, 0.5% SDS and 10 µg ml⁻¹ denatured salmon sperm DNA. The hybridized filters were washed for 1 h at 62 °C in 0.5 × SSC, 0.1% SDS and exposed to Kodak XAR film for 3 days with an intensifying screen.

The *mas* oncogene encodes a product with properties of angiotensin II receptors²⁵. A comparison of this sequence with that of the AT₁ receptor reported here reveals very little similarity between the two. An alignment of the entire sequence of both proteins reveals an overall identity of 8% that improves to 19% when only residues encompassing the hydrophobic cores of both proteins are compared. Thus it is unclear how the *mas* oncogene product relates to the vascular AT₁ angiotensin II receptor. *mas* may represent a related gene, however, as angiotensin receptor heterogeneity is now well documented pharmacologically. It is likely that nucleotide probes based on the AT₁ receptor sequence may be useful in isolating additional members of the angiotensin receptor family. As the gene family is defined, the relationship of *mas* to AT₁ and other subtypes will probably become apparent.

In addition to mediating contraction of vascular smooth muscle, angiotensin II affects renal¹¹, adrenal cortical⁵ and hepatic functions²⁶, modulates both peripheral autonomic and central nervous system activity²⁷, and is thought to have a role in development²⁸. General understanding of this critically important hormonal system has been hindered by difficulties in defining the primary structure of the receptor and cloning its gene. The results reported here should facilitate the development of insights into angiotensin receptor function, regulation, and heterogeneity. Furthermore, this clone should make it possible to isolate the human gene and to test the hypothesis that the angiotensin receptor is a candidate gene contributing to the genetic component of hypertension. □

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Impairment of mitochondrial transcription termination by a point mutation associated with the MELAS subgroup of mitochondrial encephalomyopathies

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DEFECTS in mitochondrial DNA (mtDNA) are associated with several different human diseases, including the mitochondrial encephalomyopathies. The mutations include deletions but also duplications and point mutations¹. Individuals with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) carry a common A-to-G substitution in a highly conserved portion of the gene for transfer RNA^{Leu(UUR)} (refs 2, 3). Although the MELAS mutation may be comparable to the defect in the tRNA^{Leu} gene associated with MERRF (refs 4, 5) (myoclonus epilepsy associated with ragged-red fibres), it is also embedded in the middle of a tridecamer sequence necessary for the formation of the 3' ends of 16S ribosomal RNA *in vitro*⁶. We found that the MELAS mutation results in severe impairment of 16S rRNA transcription termination, which correlates with a reduced affinity of the partially purified termination protein for the MELAS template. This suggests that the molecular defect in MELAS is the inability to produce the correct type and quantity of rRNA relative to other mitochondrial gene products.

The human mtDNA transcription termination site is located at the boundary of the genes for 16S rRNA and tRNA^{Leu(UUR)} (Fig. 1a, b), and is the major determinant controlling the relative synthesis of rRNA compared to other RNA molecules^{6,7}. Termination-competent extracts contain a factor capable of footprinting the tridecamer sequence and several of its flanking nucleotides⁸.

We have been examining mitochondrial extracts that support RNA 3'-end formation in order to begin identification of the protein(s) that are critical for this event. We have localized an active fraction from denaturing polyacrylamide gels consisting of proteins ranging in relative molecular mass from ~33,000 to 36,000 (*M_r* 33–36K), the most abundant species being a protein of ~34K (Fig. 2a). This fraction (designated mtTERM) yields reproducible positive results for termination activity as judged by gel-shift (Fig. 2b) and transcription termination (Fig. 2c) assays. Although the ~34K protein is the principal species detectable by silver staining in the gel fraction used in this study, we have not established whether there is only one or multiple proteins of similar size that represent the necessary *trans*-acting factors to effect 3'-end formation. We assume that this protein(s) represents a nuclear gene product, given that all human mtDNA reading frames of significant size have been assigned⁹ and none would encode a protein of this size.

We next tested for any alteration in transcription termination that might be ascribed to the MELAS mutation. The effect of the MELAS point mutation on mtTERM binding and function was examined using gel-shift analysis (Fig. 3a), DNase I footprinting (Fig. 3b) and, most important, runoff transcription/termination (Fig. 3c). The results obtained with wild-type template are consistent with all previous data; in particular, the efficiency of termination in the runoff/termination reaction is ~50% (Fig. 3c, lane 2). In sharp contrast, the MELAS mutation renders the template sequence ineffective in transcription termination, as judged by three separate criteria. First, the relative amount of mtTERM binding of equivalent amounts of template is only one-half that of wild type (Fig. 3a, lanes 2 and 6). As expected, competition with 0.5 molar amount of wild-type template reduces binding to wild-type fragment by 40%, whereas an equal amount of MELAS template only competes away 6%

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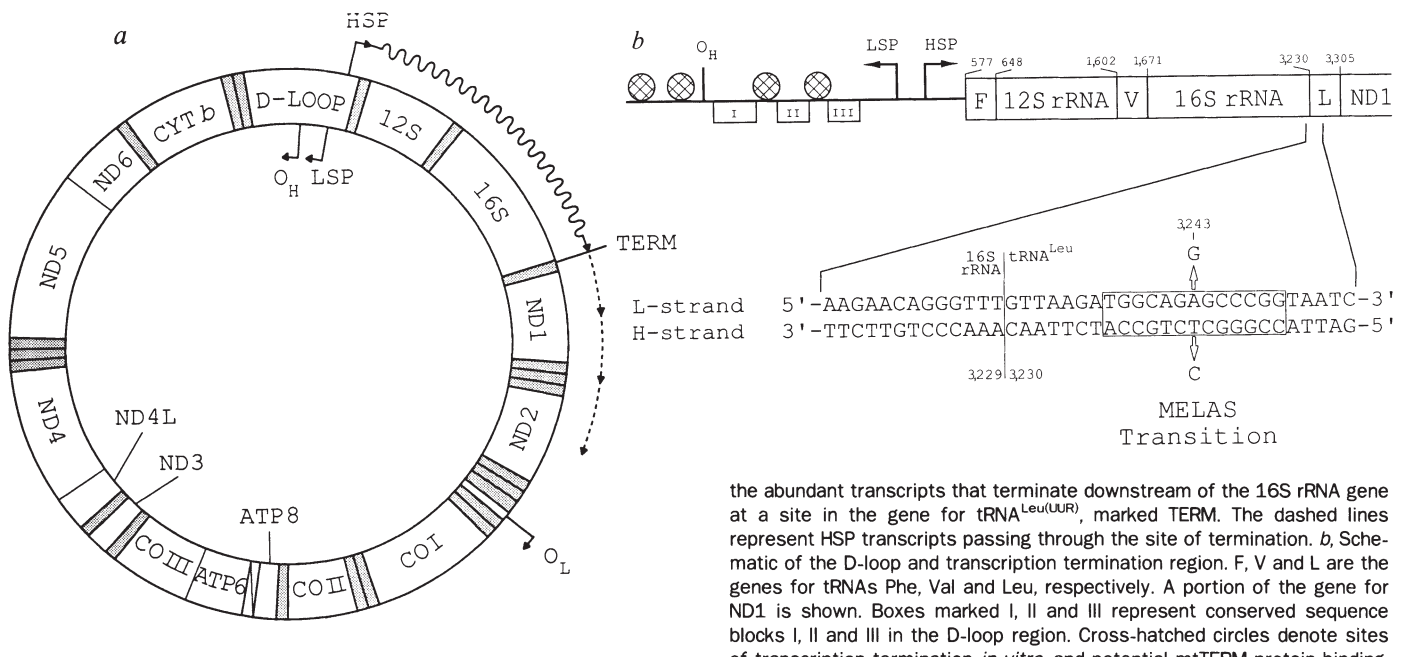
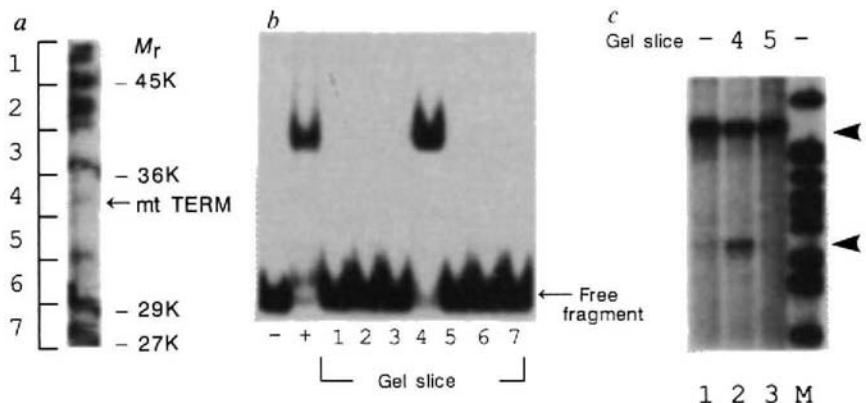


FIG. 1. *a*, Representation of human mtDNA. The shaded areas represent the 22 tRNA genes. Open regions denote control regions, rRNA genes, and protein-coding genes. These include the displacement-loop (D-loop) region, the 12S and 16S rRNA genes, and the genes for cytochrome *c* oxidase subunits I, II, and III (COI, COII, COIII), ATPase subunits 6 and 8 (ATP6, ATP8), cytochrome *b* (CYTB), and subunits 1, 2, 3, 4, 4L, 5 and 6 of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6). O_H and O_L demarcate the respective origins of heavy (H) and light (L) strand mtDNA synthesis. HSP and LSP mark the respective promoters for transcription from the H and L template strands. The wavy line originating from the HSP represents

the abundant transcripts that terminate downstream of the 16S rRNA gene at a site in the gene for tRNA^{Leu(UUR)}, marked TERM. The dashed lines represent HSP transcripts passing through the site of termination. *b*, Schematic of the D-loop and transcription termination region. F, V and L are the genes for tRNAs Phe, Val and Leu, respectively. A portion of the gene for ND1 is shown. Boxes marked I, II and III represent conserved sequence blocks I, II and III in the D-loop region. Cross-hatched circles denote sites of transcription termination *in vitro*, and potential mtTERM protein binding, in the D-loop region downstream of the LSP (ref. 6). The LSP-driven transcription templates used in this study have been constructed for maximum transcription efficiency and lack these potential termination sites. Other symbols are as described above. Illustrated below the schematic is the sequence surrounding the site of transcription termination in the gene for tRNA^{Leu(UUR)}. The boundary separating the genes for 16S rRNA and tRNA^{Leu(UUR)} is indicated by the vertical line; the box encloses the tridecamer sequence essential for transcription termination *in vitro*⁶. The open arrows and nucleotides at position 3,243 mark the site of the transition mutation⁷ associated with the MELAS type of mitochondrial encephalomyopathy^{2,3}.

FIG. 2 Identification of a mitochondrial protein fraction with gel-shift and transcription termination activities. *a*, Denaturing polyacrylamide gel fractionation of a protein fraction active in transcription termination. The peak of termination factor activity from a purified mitochondrial protein extract was electrophoresed on an SDS-polyacrylamide gel and visualized by Coomassie blue staining alongside size markers whose migrations are indicated to the right. *b*, Slices of the gel were excised as diagrammed on the left of *a* and the proteins eluted from each numbered slice were tested for the ability to retard a termination-site-containing end-labelled fragment of DNA. The (–) lane contains no added protein, and the (+) lane contains a sample of the pooled peak fractions used for gel electrophoresis. The protein-bound fragment migrates with a slower mobility than the unbound free fragment. *c*, Gel slices 4 and 5 were tested in an *in vitro* transcription assay for the ability to terminate transcription at the 16S rRNA-tRNA^{Leu(UUR)}



boundary in the presence of a mitochondrial RNA (mtRNA) polymerase fraction and DNA template containing the termination site. Lane 1 contains elution buffer only; lanes 2 and 3 contain eluted and concentrated protein from gel slices 4 and 5, respectively; lane M contains *Hpa*II-digested pBR322 DNA. The top arrow indicates the position of the runoff (260 nucleotides), the lower arrow that of the terminated transcripts (163 nucleotides).

METHODS. Mitochondria from human KB cell cultures were isolated using sucrose step gradients as described¹⁰. Mitochondria were resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF) and lysed by the addition of NaCl to 0.35 M and Triton X-100 to 1% and vigorous vortexing. Ultracentrifugation at 45,000 r.p.m. for 60 min yielded an S-130 supernatant which was diluted to 0.2 M NaCl with buffer A and loaded on a DEAE-Sephacel column. On elution with buffer A plus 0.3 M NaCl, fractions were tested for nonspecific RNA polymerase activity as assayed on poly(dA·dT) template DNA (ref. 11). Peak fractions of mtRNA polymerase activity containing mitochondrial transcription factor 1 (mtTF1) (ref. 11) and mtTERM were pooled and loaded onto a P-11 phosphocellulose column and eluted with a linear 0.3–1.0 M NaCl gradient. Fractions were assayed for nonspecific RNA polymerase activity or for termination activity

by gel shift. Active polymerase fractions were pooled and dialysed against buffer A without PMSF plus 50% glycerol in the presence of $100\text{ }\mu\text{g ml}^{-1}$ BSA. Termination fractions were pooled and loaded on a 10% SDS-polyacrylamide gel alongside protein size markers. Gel slices were processed¹² with a final dilution of 25-fold. Mobility shift analyses for termination factor binding were performed as described¹³. A typical reaction volume of $20\text{ }\mu\text{l}$ contained 10 mM Tris, 8.0 , 10 mM MgCl_2 , 1 mM DTT, $100\text{ }\mu\text{g ml}^{-1}$ BSA, and $0.1\text{--}1\text{ ng}$ of ^{32}P -end-labelled DNA fragment. All reactions contained the nonspecific competitor plasmid pGEM 3/4 at $0.5\text{ }\mu\text{g ml}^{-1}$ and $10\text{ }\mu\text{l}$ of protein renatured from a gel slice. For *in vitro* transcription/termination reactions, the renatured gel samples were diluted fourfold with buffer A without PMSF and concentrated fivefold using Amicon 10 microconcentrators. Transcription/termination reactions were performed in a final volume of $25\text{ }\mu\text{l}$ according to Fisher and Clayton^{11,14}, except that each included $4\text{ }\mu\text{Ci}$ [^{32}P]GTP, $4\text{ }\mu\text{l}$ peak mtRNA polymerase activity containing mtTF1, and $1\text{ }\mu\text{g ml}^{-1}$ of template DNA pLSPTERM containing the LSP (nucleotides 323–445 (ref. 15)), an *EcoRI* linker, and the termination region (nucleotides 3,160–3,314). Lanes 2 and 3 contain $5\text{ }\mu\text{l}$ of the appropriate renatured protein fraction.

of labelled fragment (Fig. 3a, lanes 3 and 4). Likewise, the wild-type fragment is more effective than the mutant fragment in competing away the already reduced shift produced with the MELAS fragment. Second, the strong footprint associated with binding of the mtTERM fraction to the wild-type sequence (Fig. 3b, lanes 1 and 2) is altered in the case of the MELAS mutation; the breadth and strength of protection is less and at least two sites of DNase I hypersensitivity are apparent (one within and one adjacent to the tridecamer sequence) (Fig. 3b, lanes 4 and 5). Third, in the transcription termination functional test (Fig. 3c), the MELAS mutation renders the sequence <10% as efficient as wild-type in RNA 3'-end formation, with the expected

corresponding increase in percentage of full length runoff transcripts.

These results show that the point mutation associated with MELAS causes a dramatic loss of function of the transcription termination sequence, as judged by *in vitro* analyses. The data do not, of course, address the issue of whether the MELAS mutation has any additional effect at the level of altered tRNA^{Leu(UUR)} structure and function. Regardless of this, the MELAS mutation causes a significant dysfunction of one of the few documented control regions in human mtDNA. Loss of termination would almost certainly interfere with efficient and proper synthesis of 16S rRNA (and presumably 12S rRNA as

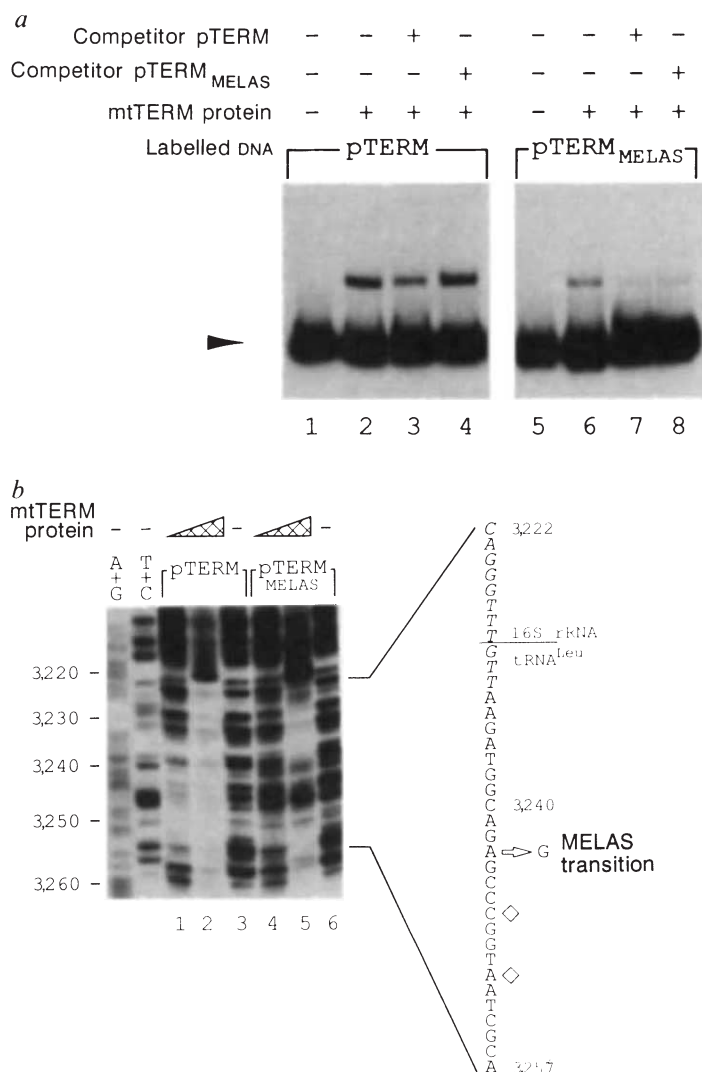


FIG. 3 Impaired gel-shift, footprinting, and transcription termination properties of mtTERM protein on a target termination site carrying the MELAS point mutation. **a**, Mobility shift of wild-type and MELAS end-labelled DNA fragments in the presence and absence of unlabelled competitor DNAs. Lanes 1–4 contain the 3'-end-labelled fragment of pTERM (containing the termination site); lanes 5–8 contain pTERM_{MELAS} (the identical fragment except for the MELAS point mutation at position 3,243). Lanes 1 and 5 have no protein; the remainder of the lanes contain 2 μ l of concentrated gel fraction 4 protein from Fig. 2a. Nonradioactive competitor DNA was added at a 0.5 molar excess relative to labelled fragment. Arrowhead indicates position of the free fragment. **b**, DNase I footprint of pTERM and pTERM_{MELAS}. Base positions are indicated to the left of the figure alongside sequencing ladders¹⁶. Lanes 1–3 represent the DNase cleavage patterns of end-labelled pTERM in the absence (lane 3) or in the presence of 4 μ l (lane 1) or 8 μ l (lane 2) of a concentrated P11 pool of termination factor activity. Lanes 4–6 show identical reactions performed on the mutant template, pTERM_{MELAS}. The sequence of the region protected by the protein in lanes

1 and 2 is indicated in brackets at the right, with the point mutation shown by an open arrow. Italics indicate the footprinted region (nucleotides 3,222–3,232) that extends beyond the published footprint⁸; this may reflect non-specific protection due to additional proteins present in this partially purified termination preparation. Protection from gel slice 4 material exhibits a footprint consistent with that published⁸ (data not shown). The 16S RNA–tRNA^{Leu} gene boundary is represented by a horizontal line. Hypersensitive bands produced on pTERM_{MELAS} fragment in lanes 4 and 5 are indicated by open diamonds. Note that the assignment of nucleotides relative to the sequencing ladders is offset by one position due to the nature of the chemical cleavage reaction which produces a fragment shorter by one nucleotide than the corresponding DNase I-cleaved fragment. **c**, Absence of transcription termination on the termination sequence containing the MELAS point mutation. Lanes 1 and 2 contain the wild-type pTERM sequence linearized by *Hind*III digestion, whereas lanes 3, 4 and 5, 6 contain the same amount and twice the amount of pTERM_{MELAS} template, respectively. All *in vitro* reactions contain added mtRNA polymerase and mtTF1; lanes 2, 3 and 5 contain 8 μ l of renatured and concentrated mtTERM protein from gel slice 4. The runoff transcript (260 nucleotides) is indicated by the upper arrow and the terminated transcript (163 nucleotides) by the lower arrow. Lane M, *Hpa*II-digested DNA pBR322.

METHODS. Plasmid pTERM was constructed by subcloning the *Bam*HI–*Hind*III fragment of pLSPTERM, encoding the LSP and transcription termination region, into pBLUESCRIPT KS+. Site-directed mutagenesis¹⁷ on single-stranded DNA with the oligonucleotide 5'-GATCTGTTAAGATGGCAGGG-CCCGGT-3' resulted in the plasmid pTERM_{MELAS}. The point mutation at nucleotide position 3,243 was confirmed by dideoxy sequencing using the T7 DNA sequencing kit (Pharmacia). Mobility shift experiments were as described in Fig. 2 with the following exceptions: reactions contained 0.05 μ g ml⁻¹ of 3'-end-labelled DNA fragment and 5 μ g ml⁻¹ nonspecific competitor DNA, pBLUESCRIPT KS (+), and were allowed to incubate at 28 °C for 20 min before loading on a polyacrylamide gel. Competitor DNA was added at 0.5 $\times$ molar excess over labelled DNA. DNase I footprinting conditions have been described^{18,19}. Runoff transcription assays are as in Fig. 2c, with the following exceptions: reactions 1–4 contained 1 μ g ml⁻¹ linearized wild-type or mutant template DNA, reactions 5–6 contained linearized mutant template at 2 μ g ml⁻¹. Protein fractions used for efficient transcription at the LSP were 2 μ l of a phosphocellulose-purified mtRNA polymerase fraction lacking mtTF1 and 2 μ l of gel-purified mtTF1. Gels were measured on the basis of c.p.m. using an AMBIS Radioanalytic Imaging System and peaks of radioactivity were integrated and compared for determining relative transcription termination or mobility shift.

well (Fig. 1)), would remove at least the major control point for regulating the relative amount of rRNA synthesis versus that of other genes encoded on this strand of mtDNA, and could conceivably impact directly on the proper processing of tRNA^{Leu(UUR)}. Furthermore, we note the occurrence of possible transcription termination sites in the D-loop control region of human mtDNA (ref. 6) (depicted in Fig. 1b). Any overproduction of mtTERM in response to the MELAS mutation might result in transcription termination and, possibly, defective replication priming and elongation within the D-loop region on both MELAS and wild-type mtDNAs. Such a dominant effect would be a fundamental aspect of the disease phenotype, given that the patients' mtDNA populations are heteroplasmic^{2,3}.

This first link between a human disease and a molecular regulatory feature of human mtDNA emphasizes the rich opportunity that these mitochondrial disorders provide for understanding the intimate details of mitochondrial biogenesis. In particular, learning the exact nature of the protein(s) contained within the partially purified 34K fraction should reveal not only the details of the termination process, but also point to a possible means of investigating or treating this disease at the nuclear gene level. □

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Demethylation of CpG islands in embryonic cells

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DNA in differentiated somatic cells has a fixed pattern of methylation, which is faithfully copied after replication. By contrast, the methylation patterns of many tissue-specific and some housekeeping genes are altered during normal development¹. This modification of DNA methylation in the embryo has also been observed in transgenic mice and in transfection experiments². Here we report the fate in mice of an *in vitro*-methylated adenine phosphoribosyltransferase transgene. The entire 5' CpG island region became demethylated, whereas the 3' end of the gene remained modified and was even methylated *de novo* at additional sites. Transfection experiments *in vitro* show that the demethylation is rapid, is specific for embryonic cell-types and affects a variety of different CpG island sequences. This suggests that gene sequences can be recognized in the early embryo and imprinted

with the correct methylation pattern through a combination of demethylation and *de novo* methylation.

To test whether DNA methylation inhibits gene expression *in vivo*, we introduced a methylated hamster adenine phosphoribosyltransferase (APRT) gene into a mouse during normal development. A 7.8-kilobase *HindIII* fragment³ was methylated *in vitro* using *HpaII* and *HhaI* methylases and the resulting construct, free of pBR sequences, was injected into fertilized mouse oocytes which were then transplanted into pseudopregnant recipients. This gave three founder mice, each of which contained multiple copies of the APRT gene.

Analysis of tail DNA showed that the construct had been considerably demethylated in all of the transgenic mice, but exclusively in the island portion of the gene. Use of the 900-base-pair (bp) *PvuII*-*EcoRI* probe, for example, showed that both H2 and M2 sites (H, GCGC; M, CCGG) within the CpG island are unmethylated (Fig. 1). Together with a second 5' probe we assayed six different CpG-sensitive restriction sites in the island region: all had been totally demethylated. By contrast, almost all sites 3' to the island within the body of the gene remained fully methylated, as indicated by hybridization with the 1,100-bp *EcoRI*-*PvuII* probe, as did several 5' flanking CpG sequences (data not shown). Two non-island *XhoI* sites, which had not been previously methylated, were methylated *de novo*, whereas a *Bst*UI sequence in the CpG island region remained completely unmethylated (Fig. 2). This methylation pattern (Fig. 1b) was also seen in DNA from many specific tissues (Fig. 1c) and from sperm, and was preserved in two generations of offspring, both in males and females (data not shown). The pattern of methylation of the exogenous gene is essentially identical to that of the endogenous hamster gene⁴, suggesting that the genomic sequence contains the information necessary to dictate its state of methylation (see bottom panel in Fig. 1a). This branding of the DNA is probably accomplished through the coordinated action of both *de novo* and demethylation activities present during early stages of mouse development.

Because it seems that transfected APRT gene is not demethylated in fibroblast cells⁵, the *in vivo* demodification could be restricted to embryonic cells. To test this possibility, we introduced an *in vitro*-methylated APRT gene into F9 teratocarcinoma cells. Six *HpaII* and *HhaI* sites in the CpG island sequence were all totally demethylated (Fig. 3a). CpG residues outside the island region remained fully modified (Fig. 3b) and, as a result, the APRT gene acquired a methylation pattern similar to that of the endogenous gene in hamster cells. Slow changes in methylation may occur artefactually in cells adapted for growth in culture^{6,7}. The island-specific demethylation in F9 cells, however, seems to be rapid and can be readily detected within 48 h after transient transfection (Fig. 3c). The reaction is also cell-type specific because it occurred in two other independently derived embryonal carcinomas, OTF9-63 and C86S1A1 (Fig. 4c), but not in mouse L-cell fibroblasts or L8 myoblasts (data not shown). We also fused *hpri*⁻ OTF9-63 cells with *tk*⁻ L cells transfected with a hamster APRT gene methylated *in vivo* at *HpaII* and *HhaI* sites, and selected hybrids by using a G418 resistance marker. In the hybrid colonies (*neo*⁺, *tk*⁺), more than 80% of the *HhaI* site (Fig. 3a), as well as others (data not shown) in the APRT promoter, was unmethylated, despite the fact that these loci are almost fully methylated before fusion or after control fusions with the *hpri*⁻ A9 fibroblast line. These experiments clearly show that the demethylation machinery in embryonic cells is dominant.

The demodification seems to be specific for CpG islands. In addition to APRT, the X-linked Pdk-1 island was demethylated in F9, but not in L-cells (Fig. 4a), and a similar demodification was seen for the CpG islands in the mouse metallothionein gene and in two different apolipoprotein genes (data not shown). By contrast, non-CpG island genes, such as insulin, remained fully methylated in embryonic cells (Fig. 4b). Because the APRT transgene was methylated *de novo* at several sites flanking the

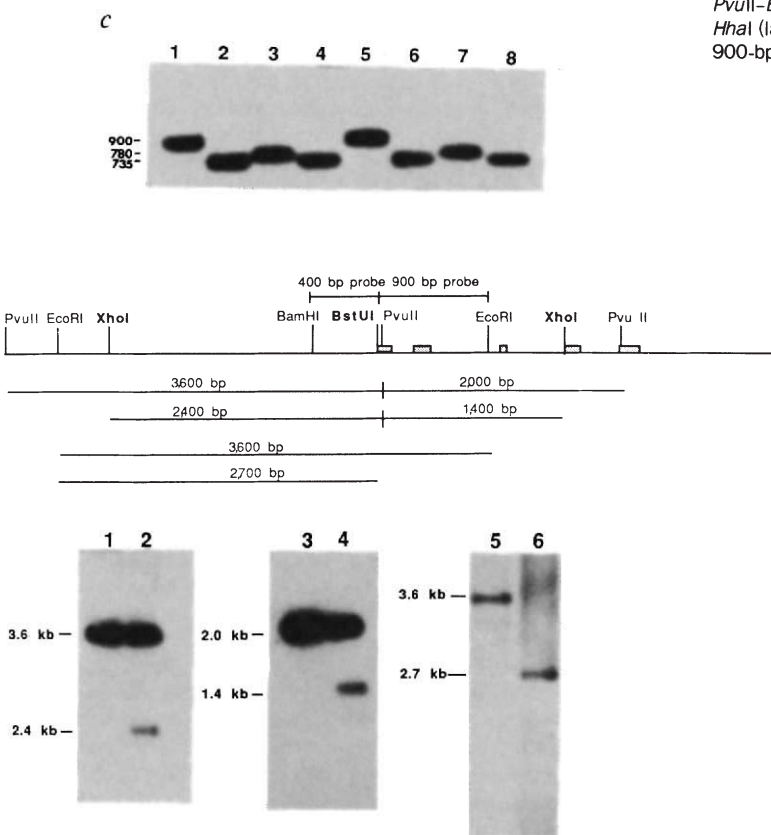
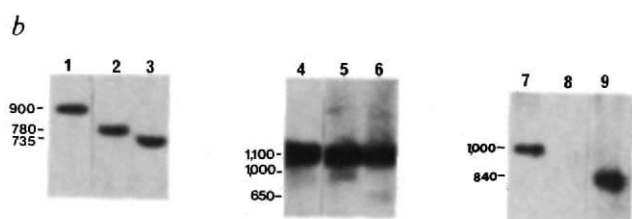
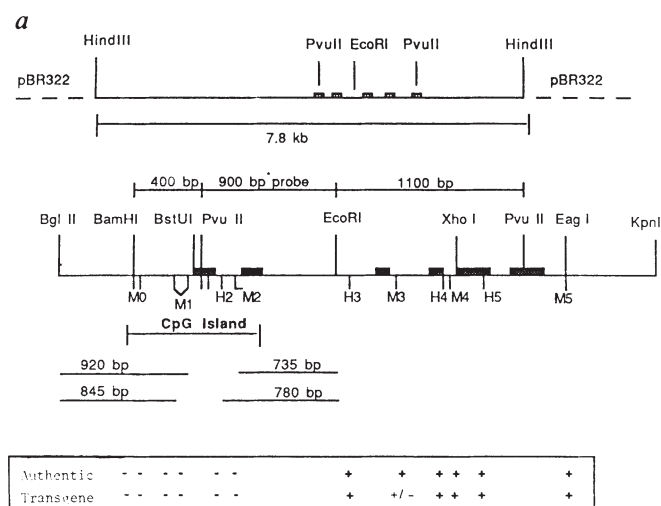


FIG. 1 Demethylation of the APRT CpG island in transgenic mice. **a**, Maps of the overall structure of the hamster APRT gene (top), and the gene in more detail (middle)³. M and H, *MspI* (*HpaII*) and *HhaI* site, respectively. The maps also show the various probes, the extent of the CpG island and the sizes of several restriction fragments. Bottom, the methylation state of the authentic gene in hamster tissues⁴ and the transgene analysed in this study. +, A site more than 80% methylated; -, a site with less than 20% modification. Modification of site (M3) in the transgene was intermediate (+/-). Restriction digestions were carried out with a fivefold excess of enzyme for 4–5 h and deemed to be effective if unmethylated sites were digested to completion. All bands are specific for the exogenous hamster APRT gene. **b**, Transgenic mice were prepared²³ from the *HindIII* fragment containing the APRT gene which had been methylated at *HpaII* and *HhaI* sites *in vitro*. Before injection, this fragment was shown to be completely methylated by testing with the restriction enzymes *HpaII* and *HhaI*⁵. DNA (3 µg) isolated from mouse tails was digested with *PvuII*–*EcoRI* alone (lane 1), and together with *HhaI* (lane 2) or *HpaII* (lane 3). After electrophoresis and Southern blotting, the filter was hybridized with the 5' 900-bp probe to analyse sites M2 and H2. This same filter was then rehybridized (lanes 4–6) with the 3' 1,100-bp probe to study the degree of methylation of sites M3, M4, H3, H4 and H5. M3 was variable and could reach 50% methylation in some animals. The same DNA (3 µg) was digested with *BglII*–*PvuII* alone (lane 7), or together with *HpaII* (lane 8) or *NciI* (lane 9), and analysed with the 400-bp *BamHI*–*PvuII* fragment (site clusters M0 and M1). That no bands were seen after *HpaII* digestion suggests that all of the M0 and M1 sites are unmethylated and that the limit digest is too small to be seen on the blot. The undermethylation of site M1 (also an *NciI* locus) is demonstrated directly in lane 9. **c**, DNA samples (3 µg) isolated from spleen (lanes 1–4) or liver (lanes 5–8) from a female F₁ transgenic mouse were digested with *PvuII*–*EcoRI* alone (lanes 1 and 5) or together with *HpaII* (lanes 2 and 6), *HhaI* (lanes 3 and 7) or *MspI* (lanes 4 and 8). Hybridization was with the 5' 900-bp probe, and this reveals the methylation state of sites M2 and H2.

FIG. 2 *De novo* methylation of the APRT gene in transgenic mice. DNA samples (5 µg) isolated from mouse tails were digested with *PvuII* (lanes 1, 3), *PvuII*–*XhoI* (lanes 2, 4), *EcoRI* (lane 5) and *EcoRI*–*BstUI* (lane 6). After electrophoresis and Southern blotting, the filters were hybridized with the 400-bp *BamHI*–*PvuII* probe (lanes 1, 2, 5, 6) or the 900-bp *PvuII*–*EcoRI* probe (lanes 3, 4). The degree of *de novo* methylation at the unique *BstUI* site (lane 6) and *XhoI* sites in the 5' flanking region (lane 2) and 3' region of the gene (lane 4) was determined in this experiment.

island, we investigated whether this *de novo* activity was also present in the embryonal carcinoma cells used in this study. The 5' *XhoI* site was methylated *de novo* in several teratocarcinomas (Fig. 4c), and similar patterns were observed for the 3' *XhoI* site (data not shown). Despite the strong *de novo* activity in C861SA1 cells, their APRT island sequences were demethy-

lated (Fig. 4c), indicating that both reactions can coexist in the same cell. Many endogenous non-island sequences become modified in the early embryo, probably during the late blastocyst stage^{8–11}. This same *in vivo* activity could account for the *de novo* modification observed in embryonic transfections^{12,13} and in transgenes^{2,14}. An island demethylation system operating

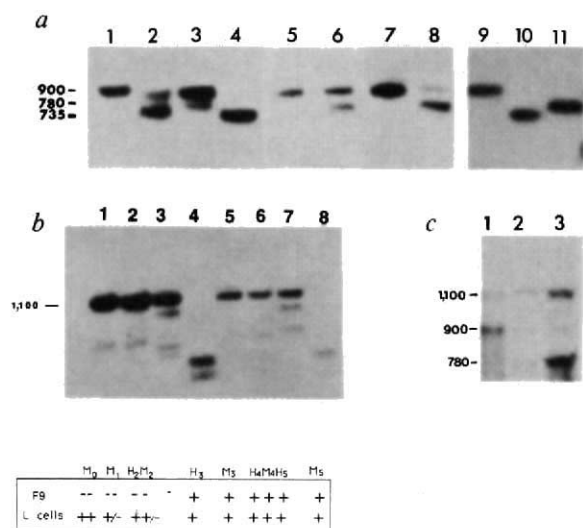
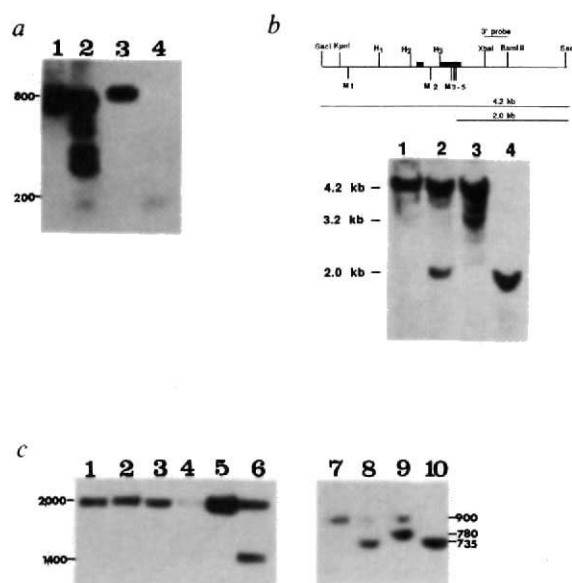


FIG. 3 Transfection of APRT in L-cells and F9 cells. *a*, Plasmid pHapT³ was methylated *in vitro* with *HpaII*-*HhaI* methylases, tested for modification and the same batch cotransfected into both L- and F9 *tk*⁻ cells using a G418 resistance vector⁵. DNA samples (10 µg) isolated from pooled colonies of transfected L-cells (lanes 1–4), F9 cells (lanes 5–8), L cell X A9 hybrids (lanes 9 and 6) and L cell X F9 hybrids (lanes 7 and 8) were digested with *PvuII*-*EcoRI* alone (lanes 1, 5, 7, 9) or together with *HpaII* (lanes 2 and 10), *HhaI* (lanes 3, 6, 8, 11) or *MspI* (lane 4). Hybridization was with the 900-bp *PvuII*-*EcoRI* probe (sites M2 and H2). Two specific restriction sites within the promoter region underwent anomalous demethylation in L-cells. Similar site-specific demethylation has been observed for other *in vitro*-methylated housekeeping genes transfected into fibroblasts²⁴. The markers on the left indicate the approximate positions of the expected bands. *b*, The same filter as in *a* was hybridized with the 3' 1,100-bp *EcoRI*-*PvuII* probe, allowing us to analyse methylation at *HpaII* sites, M3 and M4 (lanes 2 and 6) and *HhaI* sites H3, H4, and H5 (lanes 3 and 7). A summary of these and other experiments is presented below. The faint bands seen in the lower part of the blot are not derived from the hamster APRT gene and may represent slight cross-hybridization with endogenous APRT sequences. *c*, F9 *tk*⁻ cells were transiently transfected²⁵ with an *in vitro*-methylated plasmid pHapT for various times and nuclear DNA prepared and analysed by Southern blot hybridization using a mixture of the 900-bp 5' probe and the 1,100-bp 3' probe. DNA was digested with *PvuII*-*EcoRI*-*HhaI* at 0 time (lane 1), 48 h (lane 2) or 120 h (lane 3). Note that only the sites in the 5' region become demethylated, whereas the 3' CpGs remain modified in the same sample. Similar results were obtained with *HpaII* (data not shown).



during this period would explain why transgenic island sequences seem to be protected from this process^{15,16}.

After inactivation at the late blastocyst stage of development, housekeeping gene islands on the X chromosome become methylated *de novo*^{17,18}. Reactivation of the X chromosome is accomplished in primordial female germ cells before the first meiosis^{19,20}. If germ-line X-linked sequences are methylated *de novo* like their somatic counterparts, the island demethylation could be actively involved in this reactivation process. Alternatively, this enzymatic system may be involved in protecting these cells from the *de novo* methylation that occurs in the embryo at about the gastrula stage⁸. Indeed, many X-linked genes, including *Pgk-1*, are unmethylated in female primordial germ cells²¹. The existence of island demethylation in the early embryo may also help to explain the lack of X-chromosome methylation in extraembryonic tissues²², whose allocation takes place before implantation. □

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FIG. 4 Specificity of demethylation and *de novo* methylation in F9. *a*, A plasmid containing the *EcoRI*-*BamHI* fragment from the 5' end of the human *Pgk-1*²⁶ gene was methylated *in vitro* and inserted into *tk*⁻ L cells (lanes 1 and 2) or F9 cells (lanes 3 and 4) by DNA-mediated gene transfer. DNA (10 µg) from mass cultures was digested with *EcoRI*-*BamHI* alone (lanes 1 and 3) or together with *HpaII* (lanes 2 and 4). Hybridization was with a human-specific probe spanning the entire 800-bp *Pgk-1* fragment. Despite this fragment having eight *HpaII* sites, over 50% of the molecules remained completely methylated in L cells. *b*, Plasmid pIns-1 containing the complete rat insulin-1 gene²⁷ was methylated *in vitro* with *HpaII*-*HhaI* methylases and transfected into F9 cells. DNA samples (10 µg) isolated from pooled colonies were digested with *SacI* alone (lane 1) or together with *HpaII* (lane 2), *HhaI* (lane 3) or *MspI* (lane 4). Hybridization was with the 400-bp *XbaI*-*BamHI* probe (shown on the map). The degree of modification at *HpaII* (M) and *HhaI* (H) sites were analysed in this experiment. Exons are shown as hatched boxes. *c*, Several embryonic carcinoma cell lines were transfected with the *in vitro*-methylated APRT gene construct and analysed for demethylation and *de novo* methylation. DNA (10 µg) from C86S1A1 (lanes 1 and 2), OTF9-63 *hprt*⁻ (lanes 3 and 4) and F9 *tk*⁻ (lanes 5 and 6) were digested with *PvuII* alone (lanes 1, 3, 5) or together with *XhoI* (lanes 2, 4, 6). Hybridization with the 900-bp *PvuII*-*EcoRI* probe reveals methylation at the 3' *XhoI* site (see Fig. 2). DNA from C86S1A1 was also digested with *PvuII*-*EcoRI* alone (lane 7) or together with *HpaII* (lane 8), *HhaI* (lane 9) or *MspI* (lane 10) and hybridized to the same probe (sites M2 and H2).

Dephosphorylation and activation of a p34^{cdc2}/cyclin B complex *in vitro* by human CDC25 protein

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OOCYTES arrested in the G2 phase of the cell cycle contain a p34^{cdc2}/cyclin B complex which is kept in an inactive form by phosphorylation of its p34^{cdc2} subunit on tyrosine, threonine and perhaps serine residues (see refs 1 and 2 for review). The phosphatase(s) involved in p34^{cdc2} dephosphorylation is unknown, but the product of the fission yeast *cdc25⁺* gene^{3,4}, and its homologues in budding yeast⁵ and *Drosophila*⁶ are probably positive regulators of the transition from G2 to M phase. We have purified the inactive p34^{cdc2}/cyclin B complex from G2-arrested starfish oocytes. Addition of the purified bacterially expressed product of the human homologue of the fission yeast *cdc25⁺* gene⁷ (p54^{CDC25H}) triggers p34^{cdc2} dephosphorylation and activates H1 histone kinase activity in this preparation. We propose that the *cdc25⁺* gene product directly activates the p34^{cdc2}-cyclin B complex.

To investigate the mechanism of *cdc2* kinase activation, we purified the inactive p34^{cdc2}/cyclin B complex, to be used as a substrate in screening assays for the putative p34^{cdc2} phosphatase activity. Isolation of the native p34^{cdc2}-cyclin B complex from G2 oocytes is difficult because it is 'spontaneously' activated when ATP is removed from crude homogenates⁸⁻¹⁰ owing to dephosphorylation of its p34^{cdc2} subunit (J.C.L. and M.D. unpublished observations). We purified the native complex in its inactive form from G2-arrested starfish oocytes by adding ATP and vanadate (both 1 mM) to homogenates and buffers during the two first steps of the purification. Except for this modification, purification was as previously described for the active complex^{11,12}. Briefly, the material which eluted at 200 mM NaCl from a DEAE-cellulose column was applied to a p13^{suc1}-Sepharose column. The inactive p34^{cdc2}/cyclin B complex was eluted with p13^{suc1} in excess, and final purification was on a Mono-S column. The p34^{cdc2}/cyclin B complex was recovered as a single peak eluting around 300 mM NaCl. Besides cyclin B (p47) and p34^{cdc2}, nothing could be detected by staining with Coomassie blue, after separation by SDS-PAGE (Fig. 1a). There were some minor proteins, including one with relative molecular mass 13,000 (*M_r* 13K) and others with *M_r* ranging from 70 to 120K when the consecutive fractions throughout the Mono-S peak were analysed by silver-staining (Fig. 1b). No additional component could be detected by autoradiography either when the inactive complex was iodinated in the presence of ¹²⁵I and chloramine T or when it was prepared from G2-arrested oocytes preloaded with ³⁵S-methionine (data not shown).

The products of the fission yeast *cdc25⁺* gene^{3,4} and its homologues MIH1 in budding yeast⁵ and the *string* gene product in *Drosophila*⁶, are positive regulators of the transition from G2 to M phase. In fission yeast *cdc25*-defective mutants were rescued by a human protein tyrosine phosphatase¹³. The mutation *cdc2*-F15, whose product could not be phosphorylated on tyrosine, bypasses the requirement for p80^{cdc25} function¹⁴. This supports the view that the *cdc25⁺* gene product is involved in regulating tyrosine dephosphorylation and activation of p34^{cdc2}/cyclin B kinase, although tyrosine dephosphorylation was not sufficient for p34^{cdc2}/cyclin B activation in higher eukaryotes^{15,16}. G2-arrested starfish oocytes contain a homologue of the *cdc25⁺* gene product (A.P., J.C.L., J.C.C. and M.D., manuscript in preparation) not detected in the purified inactive

p34^{cdc2}-cyclin B complex. We thus investigated its effect on the highly purified and inactive p34^{cdc2}-cyclin B complex prepared from starfish oocytes. We used bacterially expressed and fast protein liquid chromatography (FPLC)-purified human p54^{CDC25H} (Fig. 2b). The p54^{CDC25H} was found to induce a dramatic stimulation of the highly purified p34^{cdc2}-cyclin B kinase (Fig. 2a). Activation was associated with a shift of the p34^{cdc2} subunit towards a form of higher mobility on polyacrylamide gels (Fig. 2c left), indicating that dephosphorylation had occurred. This was confirmed using specific antibodies directed against phosphotyrosine (Fig. 2c right). When completely dephosphorylated, the *in vitro*-activated protein kinase reached a specific activity similar to that of the *in vivo*-activated protein kinase prepared from starfish oocytes at first meiotic metaphase¹¹ (about 8 μmol P transferred to H1 histone per min per mg proteins). Dephosphorylation occurred even in the absence of ATP, and therefore did not require prior phosphorylation of either cyclin B or the *cdc25⁺* gene product. A form of p34^{cdc2} with intermediate mobility was sometimes detected during activation, indicating that dephosphorylation was not limited to the removal of a single phosphoryl group on a tyrosine residue. This was most obvious when incubated with 1 mM vanadate, which depressed the rate of p34^{cdc2} dephosphorylation (Fig. 2d). This form of intermediate mobility could also be detected with anti-phosphotyrosine antibodies. A single phosphotyrosine (at Tyr 15) has been mapped on p34^{cdc2} both in fission yeast¹⁴ and chicken¹⁷, and is also presumed to exist in starfish, suggesting that Tyr 15 phosphorylation is not necessarily the first event in the process of p34^{cdc2}/cyclin B activation. It is unusual for a phosphatase to catalyse dephosphorylation of both phosphotyrosine and phosphothreonine/phosphoserine residues. But a protein sharing amino-acid sequence identity with the protein tyrosine phosphatases was shown to hydrolyse substrates containing phosphotyrosine and phosphoserine¹⁸. It was also inhibited by vanadate. Dephosphorylation of p34^{cdc2} and H1 histone kinase activation following p54^{CDC25H} addition are extremely rapid processes, taking less than 45 s at 0°C (Fig. 3a). Although stoichiometry of the reaction cannot be specified, because the proportion of correctly refolded molecules after p54^{CDC25H} solubilization from inclusion bodies in guanidine hydrochloride is not known, diluted solutions only partially activate protein kinase activity, even after long-term incubation

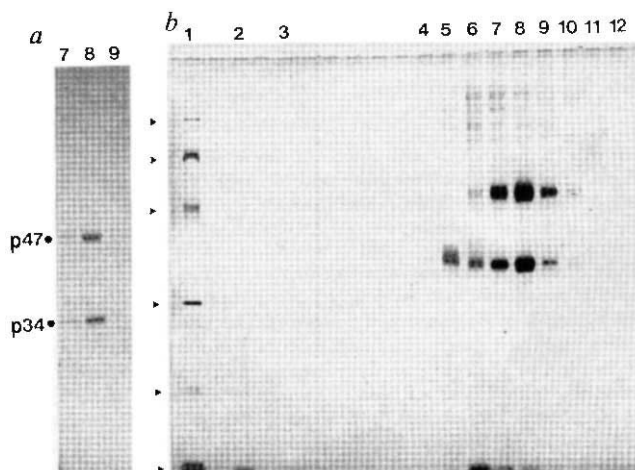


FIG. 1 Analysis by gel electrophoresis under denaturing conditions of the highly purified and inactive p34^{cdc2}/cyclin B complex isolated from G2-arrested starfish oocytes. a, Coomassie-blue staining of the three main peak fractions eluted from the Mono-S column showing cyclin (p47) and p34^{cdc2}. b, Silver staining of the consecutive fractions (lanes 4-12) throughout the peak. Marker proteins, including phosphorylase b (94K), albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20.1K) and α -lactalbumin (14.4K) were run on the left (arrowheads), using the following amounts for each marker: 50 ng (lane 1), 5 ng (lane 2) and 0.5 ng (lane 3).

(Fig. 3b). The possibility that the purified p54^{CDC25H} preparation might be contaminated by a phosphatase of bacterial origin was ruled out by the following controls. First, H1 histone kinase activation was dramatically reduced when p54^{CDC25H} was specifically immunodepleted with a polyclonal antiserum, and no activation occurred when the inclusion bodies preparation did not contain p54^{CDC25H} (Fig. 2a). Second, no phosphatase activity towards either para-nitrophenyl-phosphate (PNPP) or ³²P-labelled phosphorylase a was detected in the p54^{CDC25H} preparation. Finally, bacterially produced human p34^{CDC2H} was phosphorylated *in vitro* with baculovirus-produced pp60^{c-src}. After cyanogen bromide degradation, which released several fragments including a 32 amino acid peptide from the N terminus, and immunoprecipitation with an affinity-purified antibody against the peptide MEDYTKIEKIGEG (single-letter amino-acid code), corresponding to the N terminus of p34^{CDC2H}, a tyrosine-phosphorylated fragment of *M_r* 3.35K was detected indicating that the tyrosine phosphorylation was between residues 1 and 32. This ³²P-phosphorylated p34^{CDC2H} was not dephosphorylated upon addition of p54^{CDC25H} preparation (data not shown).

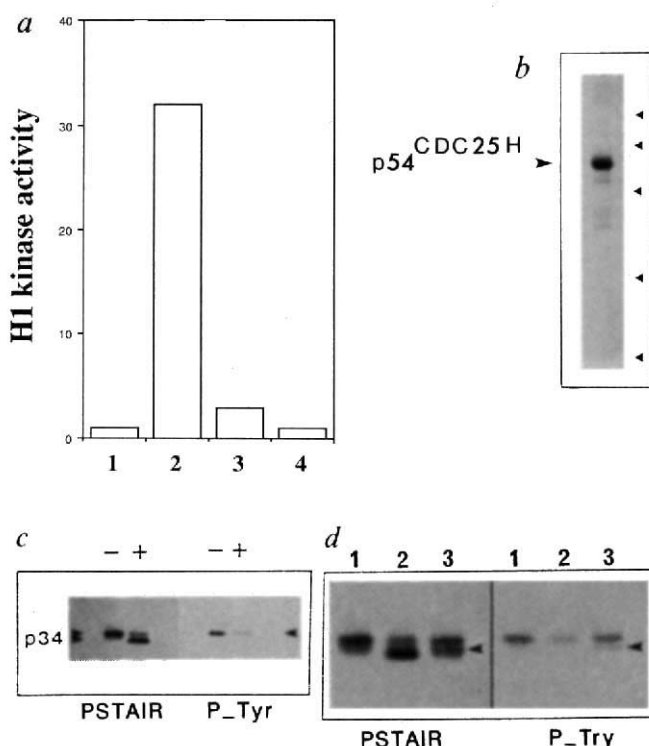
As neither p54^{CDC25H} nor its fission yeast homologue p80^{cdc25} bear much similarity to protein phosphatases^{4,7} apart from a weak homology to conserved sequences in that active site of cytoplasmic phosphotyrosine phosphatases (PTPases) (FLXK-(12)-IVXHCXXXXR)¹⁸, the above results suggest that a phosphatase activity, able to remove inhibitory phosphoryl groups from tyrosine and other residues (presumably phosphothreonine and phosphoserine, see ref. 19) was still associated with the p34^{cdc2}/cyclin B complex, despite extensive purification. Thus residual contaminants were eliminated, using immunoaffinity on affinity-purified antibodies against bacterially produced starfish cyclin B, cross-linked to protein A-Sepharose²⁰. The starting material was the peak fraction of the highly purified

p34^{cdc2}-cyclin B complex (lane 8 Fig. 1b), and soybean trypsin inhibitor was added as a carrier to avoid possible unspecific binding to the matrix. Immobilized anti-cyclin B antibodies specifically and almost quantitatively depleted the Mono-S peak fraction from the p34^{cdc2}/cyclin B complex, and all but one of the copurified proteins were left in the supernatant. The residual copurified protein (apparent *M_r* ~74K: p74), not removed in such relatively stringent conditions (300 mM NaCl; 0.1% Tween-20), was hardly detected by silver staining (arrow, Fig. 4a) and not at all by staining with Coomassie blue (data not shown). As estimated by scanning the silver stained gels (Fig. 4b), there was less than 0.5% of the amount of p34^{cdc2}/cyclin B complex. The p74 does not peak exactly with p34^{cdc2}-cyclin B on elution from the Mono-S column (see Fig. 1b). The p54^{CDC25H} still induced H1 histone kinase activation when added to the immuno-adsorbed p34^{cdc2}/cyclin B complex (Fig. 4c), and almost no H1 histone kinase activity was detected in the non-retained fraction, even after the *cdc25*⁺ gene product was added.

We cannot totally eliminate the possibility that p74 carries the phosphatase activity which, upon stimulation by the *cdc25*⁺ gene product, removes the inhibitory phosphoryl groups from p34^{cdc2}, though this appears very unlikely. Indeed any heterodimer has to be associated at least once transiently with p74 before all p34^{cdc2} subunits could be dephosphorylated. Therefore the rate of dephosphorylation would depend on the collision probability between p74 and heterodimers. In contrast to this, we found that the rate of p34^{cdc2} dephosphorylation did not increase significantly when p74 concentration was increased ~threefold with respect to p34^{cdc2}/cyclin B complex. This was demonstrated (Fig. 3a) by comparing the rate of p34^{cdc2} dephosphorylation and the resulting H1 histone kinase activation when p54^{CDC25H} was added either to the p34^{cdc2}/cyclin B peak fraction alone (lane 8, Fig. 1) or mixed with a fraction 2.5-fold enriched in p74 (lane 6, Fig. 1). Moreover, we estimated that if p74 is a

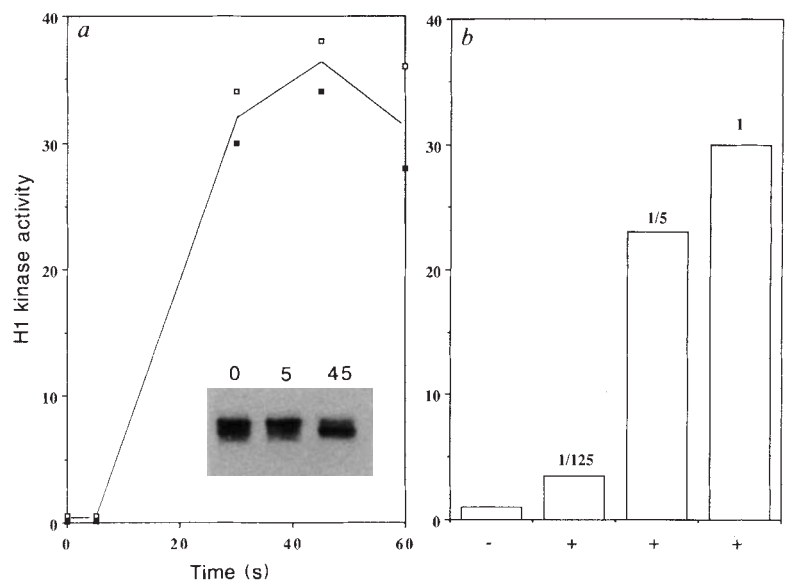
FIG. 2 The *cdc25*⁺ gene product triggers dephosphorylation and activation of the highly purified p34^{cdc2}/cyclin B complex. **a**, Bacterially expressed p54^{CDC25H} was added (2) or not (1) to the peak fraction of the p34^{cdc2}/cyclin B preparation (shown in Fig. 1, lane 8), and H1 histone kinase activity was measured 5 min later (basal H1 histone kinase activity is taken as unit). **3**, Same experiment as in (2), but the p54^{CDC25H} was first immunodepleted with a polyclonal antiserum against p54^{CDC25H}. **4**, Control experiment: the material solubilized from inclusion bodies prepared from the same strain of bacteria, but expressing a protein unrelated to p54^{CDC25H} was added to the peak fraction. **b**, Coomassie blue staining of the bacterially produced p54^{CDC25H} after solubilization from inclusion bodies. Positions of markers with *M_r* 94, 67, 43, 30, 20.1K are indicated by arrowheads (on the right). **c**, Immunoblots of the peak fraction treated either with affinity-purified polyclonal antibody against the conserved EGVPSTAIRISLLKE (PSTAIR) peptide of p34^{cdc2} homologues or an affinity-purified antibody against phosphotyrosine (P-Tyr); before addition of p54^{CDC25H} (–), 5 min after addition of p54^{CDC25H} (+). **d**, Same experiment as in **c**, except that p54^{CDC25H} was added in the presence (lane 3) or absence (lane 2) of 1 mM vanadate. Lane 1, before p54^{CDC25H} addition. Arrowheads point to the form of p34^{cdc2} with intermediate mobility, which indicates that p54^{CDC25H}-induced dephosphorylation is not limited to the removal of a single phosphoryl group on a tyrosine residue.

METHODS. p54^{CDC25H} was produced in *Escherichia coli*, expressing the full length human protein (see ref. 7) under control of T7 promoter, inducible with isopropylthiogalactoside (IPTG). Inclusion bodies were solubilized in 9 M guanidine hydrochloride made in 40 mM Hepes, 5 mM EDTA, 2 mM DTT at pH 8.5, then dialysed extensively against 40 mM NaHCO₃, 0.5 M NaCl, 2 mM DTT at pH 7.4 before freezing at –70 °C. Histone H1 kinase activation was triggered by mixing 1 volume of the dialysed solution (10–50 µg protein per ml) with 1 volume of the peak fraction of p34^{cdc2}/cyclin B preparation eluted from the Mono-S column (1–5 µg protein per ml), stored at –70 °C. After 5 min at 25 °C, H1 histone kinase activity was measured as previously described¹¹, by adding 2 µl of the above mixture into 40 µl of a solution containing 1 mg ml^{–1} H1 histones (Boehringer) 200 µM ATP (50 c.p.m. pmol^{–1}), 10 mM MgCl₂ in 20 mM Hepes at pH 7.4. The antiserum used in the experiment shown in **a** lane 3 was prepared in rabbit against the bacterially produced p54^{CDC25H} protein eluted from SDS-polyacrylamide gels. The antiserum was first adsorbed on protein A-Sepharose, then used to immunodeplete the p54^{CDC25H} preparation. One volume of the non-



retained material was added to 1 volume of p34^{cdc2}-cyclin B for assay of H1 histone kinase activation. No decrease in the extent of H1 histone kinase activation was observed when a pre-immune serum was adsorbed on protein A-Sepharose in place of the anti-p54^{CDC25H} antiserum (data not shown). Immunoblots were made on nitrocellulose sheets after separating proteins by SDS-PAGE (12.5% polyacrylamide). Alkaline phosphatase-conjugated anti-rabbit IgG antibodies were used for all experiments.

FIG. 3 *a*, Kinetics of $p34^{cdc2}$ dephosphorylation and H1 histone kinase activation at 0 °C, following addition of $p54^{cdc25H}$ to the highly purified $p34^{cdc2}$ /cyclin B complex. For H1 histone kinase assays, the kinetics of activation was monitored either when $p54^{cdc25H}$ was added to the peak fraction alone (■) or when it was added to a mixture (□) of the peak fraction (lane 8, Fig. 1) with a fraction enriched in $p74$ (lane 6, Fig. 1). *b*, Effect of diluting the $p54^{cdc25H}$ preparation (five and 125-fold) on the extent of H1 histone kinase activation. For both experiments (*a* and *b*) H1 histone kinase in the absence of added $p54^{cdc25H}$ (—) was taken as unit. METHODS. For the kinetic experiment, a mixture containing either 13 μ l of peak fraction (lane 8, Fig. 1) and 13 μ l of a fraction 2.5-fold enriched in $p74$ (lane 6, Fig. 1) or 13 μ l of peak fraction and 13 μ l of the corresponding buffer, were added to 26 μ l of the $p54^{cdc25H}$ preparation and preincubated at 0 °C. At the indicated times after mixing, either 15 μ l aliquots were taken and directly fixed in Laemmli's sample buffer for immunoblots or 1.5 μ l aliquots were transferred in 40 μ l of the cocktail used for H1 histone kinase (this prevented almost completely further activation of the $p34^{cdc2}$ /cyclin B complex). Histone H1 kinase assay was run at 25 °C as described in the legend to Fig. 2. Samples in Laemmli's sample buffer (collected at 0, 5 and 45 s) were run on SDS-polyacrylamide gels, transferred to nitrocellulose and probed with the PSTAIR antibody, as described in the legend to Fig. 2. The dilution experiment (*b*) was run at 25 °C for both activation with $cdc25$ and kinase assay. Kinase assays were performed 7 min after mixing 2 μ l



of peak fraction with 2 μ l of each $p54^{cdc25H}$ solution, by diluting 20-fold the mixture with the cocktail used for H1 histone kinase assays.

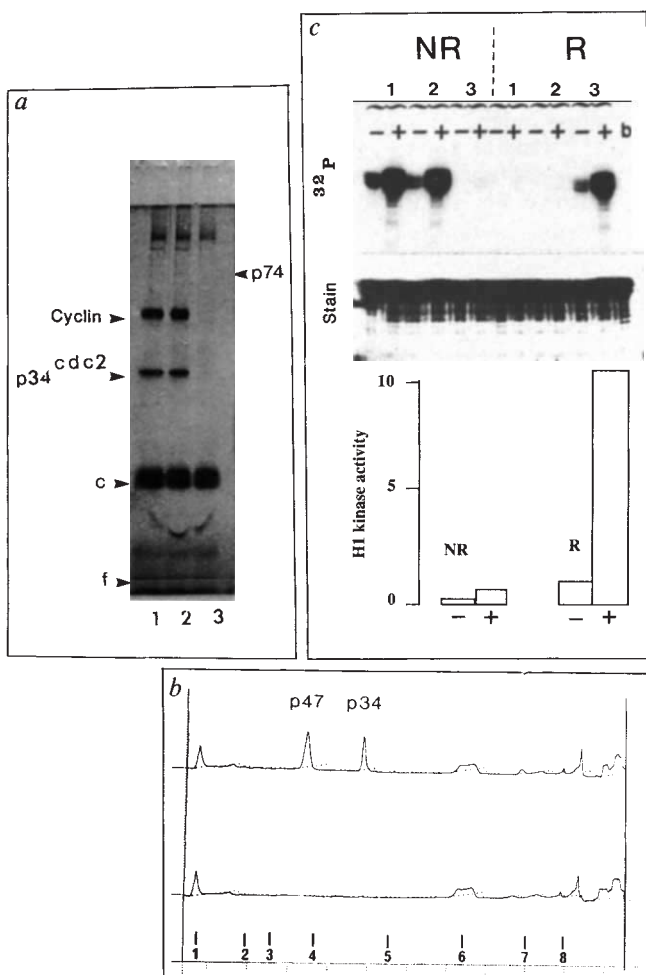


FIG. 4 The $cdc25^+$ gene product still induces H1 histone kinase activation after removal of most minor components from the highly purified $p34^{cdc2}$ /cyclin B complex. *a*, The peak fraction of the highly purified $p34^{cdc2}$ /cyclin B complex (Fig. 1*b*, lane 8) was treated with different immunoaffinity matrices. These were prepared by cross-linking to protein A-Sepharose either affinity-purified antibodies against bacterially-produced starfish cyclin B, or the same amount of unrelated immunoglobulins taken from two different rabbits (controls). Soybean trypsin inhibitor was added as a carrier (*c*) to check that there was no unspecific binding to the matrix. Both the retained and the non-retained proteins were separated by gel electrophoresis and analysed by silver staining. Although analysis of the retained proteins demonstrated the lack of unspecific binding to the immunoaffinity matrix (in contrast to $p34^{cdc2}$ and cyclin B, the carrier protein was not detected in this fraction), this part of the experiment is not shown because in Laemmli buffer immunoglobulins were released to some extent. Lanes 1 and 2, Supernatants from the two control matrices. Lane 3, Supernatant from the anti-cyclin B matrix. *b*, Scanning of lane 2 (top line) and lane 3 (bottom line) of the silver-stained gel shown in *a*. 1, Boundary between stacking and separating gel; 2–7 position of molecular mass markers (94K, 67K, 43K, 30K, 20.1K, 14.4K, respectively); 8, migration front. *c*, H1 histone kinase activities of the supernatants (NR, non-retained) and pellets (R, retained) after treatment of the highly purified $p34^{cdc2}$ /cyclin B with the control matrices (lanes 1 and 2) or the anti-cyclin B matrix (lane 3). Without $p54^{cdc25H}$ addition (—); with $p54^{cdc25H}$ addition (+); *b*, blank (only the radioactive mix used for H1 histone kinase assay was loaded). Top, autoradiography of the dried gel shown in the middle. Bottom, histogram showing the relative H1 histone kinase activities of the proteins retained (R), or not (NR), on the anti-cyclin B matrix, as assayed in the presence (+) or absence (—) of $p54^{cdc25H}$.

process *in vitro*. The $cdc25^+$ gene product may be a novel phosphatase that only recognizes the $p34^{cdc2}$ /cyclin complex. Alternatively it may merely interact with this complex, unmasking a latent 'phosphatase' activity of cyclin, or else of $p34^{cdc2}$ itself. Any of these possibilities would explain why genetic analysis has failed to identify any classical protein phosphatase as a mitotic inducer. □

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phosphatase, its specific activity would be at least 30 μ moles of phosphate removed per min per mg protein at 0 °C, which is much higher than any known protein phosphatase (see ref. 21 for review). Therefore we favour the hypothesis that the $cdc25^+$ gene product acts directly on the $p34^{cdc2}$ /cyclin B complex to induce its activation, and that nothing else is involved in this

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Identification of a G1-type cyclin *puc1*⁺ in the fission yeast *Schizosaccharomyces pombe*

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IN rapidly growing cells of the budding yeast *Saccharomyces cerevisiae*, the cell cycle is regulated chiefly at Start, just before the G1–S boundary¹, whereas in the fission yeast *Schizosaccharomyces pombe*, the cycle is predominantly regulated at G2–M (ref. 2). Both control points are present in both yeasts, and both require the p34^{cdc2} protein kinase^{3–5}. At G2–M, p34^{cdc2} kinase activity in *S. pombe* requires a B-type cyclin in a complex with p34^{cdc2} (refs 6–14); this complex is the same as MPF (maturation promoting factor^{15,16}). The p34^{cdc2} activity at the G1–S transition in *S. cerevisiae* may be regulated by a similar cyclin complex, using one of the products of a new class of cyclin genes (*CLN1*, *CLN2* and *WHI1* (*DAF1/CLN3*)) (refs 17–22). At least one is required for progression through the G1–S phase, and deletion of all three leads to G1 arrest¹⁹. *WHI1* was isolated as a dominant allele causing budding yeast cells to divide at a reduced size²¹ and was later independently identified as *DAF1*, a dominant allele of which rendered the cells refractory to the G1-arrest induced by the mating pheromone α -factor²². The dominant alleles are truncations thought to yield proteins of increased stability, and the cells are accelerated through G1 (refs 20, 22). Without *WHI1* function, the cells are hypersensitive to α -factor²², enlarged and delayed in G1 (refs 20, 22). Heretofore, this G1-class of cyclins has not been identified in other organisms. We have isolated a G1-type cyclin gene called *puc1*⁺ from *S. pombe*, using a functional assay in *S. cerevisiae*. Expression of *puc1*⁺ in *S. pombe* indicates that it has a cyclin-like role in the fission yeast distinct from the role of the B-type mitotic cyclin.

We reasoned that, given the functional complementation between the fission yeast and budding yeast p34^{cdc2} protein kinase, a G1-type cyclin from fission yeast would probably interact properly with the budding yeast regulatory system. Moreover, we assumed such a gene, if overexpressed, would also convey resistance to α -factor arrest to a strain already deficient in *WHI1* function. We therefore transformed an appropriate *S. cerevisiae* strain with a *S. pombe* complementary DNA library, expressed in a high-copy *S. cerevisiae* vector²³. One clone was isolated that showed plasmid-linked phenotypes of increased α -factor resistance and small cell size. We named this gene *puc1*⁺, for *pombe* unidentified cyclin.

The *puc1*⁺ cDNA was sequenced, and its expression in *S. pombe* verified by northern blot analysis (Fig. 1). The cDNA was 1,295 nucleotides long, which corresponds with the observed RNA size. The predicted protein product of relative molecular mass 40,000 was compared with known cyclin sequences. The *puc1*⁺ cDNA contains a region of similarity to the cyclin box regions of *WHI1*, *CLN1* and *CLN2*, including some distinct sequence features found only in the G1-type cyclins (Fig. 2). Also *puc1*⁺ shows more similarity to the A-type cyclins than do the *S. cerevisiae* genes. There is little similarity between the cyclins outside the cyclin box region.

We characterized the effects of *puc1*⁺ expression in *S. cerevisiae*, using the original cloning vector where *puc1*⁺ is constitutively expressed by the *ADH* promoter (ref. 23, and Fig. 3). Flow cytometry and microscopy of $\Delta whi1$ cells overexpressing *puc1*⁺ demonstrated that they are distinctly smaller than the same cells transformed with a control plasmid. This is similar to the findings with the *S. cerevisiae* *WHI1* gene^{20,22}, where overexpression leads to a reduced cell size. As shown by the distribution of cellular DNA content, there is only a very slight acceleration of cells through the G1 phase of the cycle. But striking acceleration through G1 in *S. cerevisiae* occurs only when the dominant alleles of the *WHI1* gene are overexpressed^{20,22}. Therefore we conclude that *puc1*⁺ behaves analogously to *WHI1* when expressed in *S. cerevisiae*, suggesting that *puc1*⁺ is acting like a G1 cyclin in budding yeast. We verified this by expressing UAS_{ADH}-*puc1*⁺ in a *S. cerevisiae* strain (relevant genotype $\Delta cln1 \Delta cln2 whi1::LEU2$ YCP[UAS_{GAL}-*WHI1*]) in which viability is conditional and galactose-dependent. The *puc1*⁺ gene could maintain viability of this strain on glucose medium, confirming that it can fulfill the role of a G1 cyclin in *S. cerevisiae*. But growth rate was reduced relative to cells expressing the endogenous *WHI1* gene; the doubling time of the strain expressing UAS_{ADH}-*puc1*⁺ in rich glucose medium was approximately 6 h compared with 4½ h for cells expressing UAS_{GAL}-*WHI1* in rich galactose medium galactose.

We next characterized the role of *puc1*⁺ in *S. pombe*. A gene disruption proved technically difficult, but we examined the effects of *puc1*⁺ overexpression. We placed *puc1*⁺ under the control of the *ntm1*⁺ promoter, which is repressed by thiamine²⁴. As normal, rapidly-growing *S. pombe* cells have a very short G1 (because Start is completed immediately on exit from mitosis), we did not expect to observe significant effects upon the G1 phase of the cell cycle. Although increased dosage of the wild-type *WHI1* allele in *S. cerevisiae* reduces cell size by acceleration through G1 (ref. 20), high overexpression of *WHI1* under the strong *GAL1* promoter leads to cell enlargement²⁵. There was a response similar to this latter phenotype with overexpression

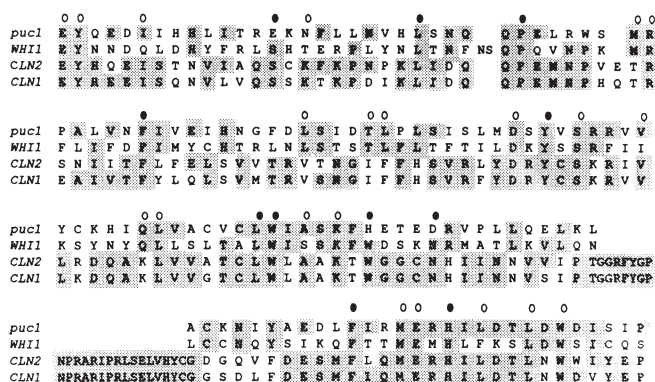


FIG. 2 Comparison of the cyclin box regions of the predicted proteins of *puc1*⁺ (starting with residue 95), *CLN1* (residue 37), *CLN2* (residue 39) and *WHI1* (residue 73). Identical residues are shaded. ○, Residues that are highly conserved in other cyclins. ●, Residues that distinguish in the G1-type cyclins from the other classes. There is little similarity in the sequences outside the cyclin box (not shown). Single-letter amino-acid code used.

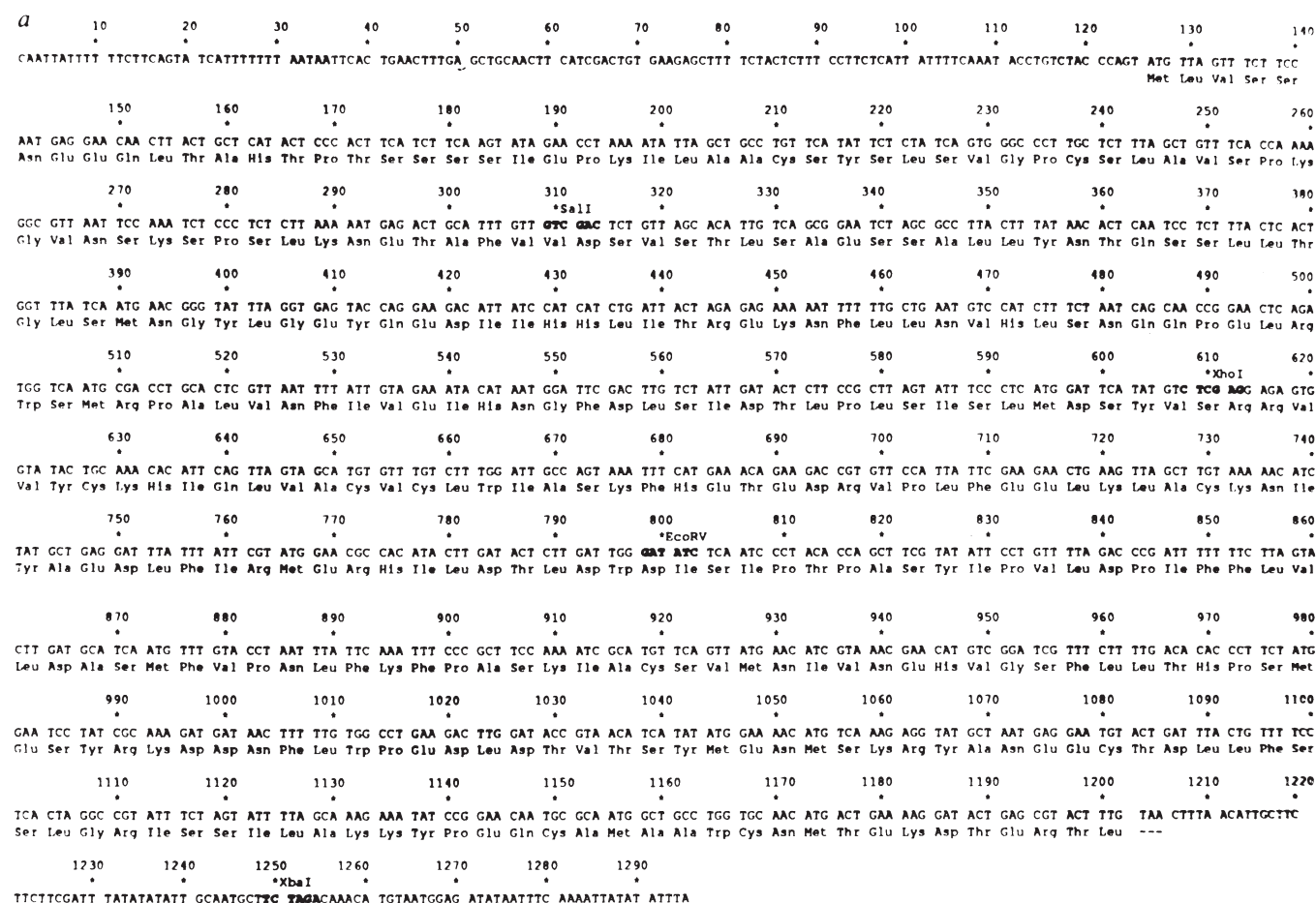
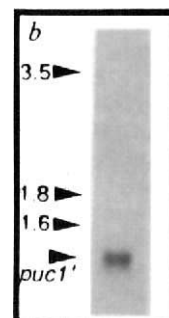


FIG. 1 Sequence and expression of *puc1*⁺. *S. cerevisiae* strain II32 ΔHpXB (relevant genotype *MATa bar1 Δdaf1* (*whi1-cln3*) *ura3*) was transformed to uracil prototrophy with an *S. pombe* cDNA library in a *S. cerevisiae* high-copy expression vector²³. Transformants (8,000) were scraped down, replated on YEPD plates containing 10⁻⁷ M α-factor (Sigma), and screened for improved growth. One clone was isolated that showed a plasmid-linked phenotype. The plasmid was isolated as described in ref. 28, transformed into *Escherichia coli* strain MC1061, and isolated by standard means. **a**, Sequence and predicted protein of the 1.3-kilobase (kb) fragment containing the *puc1*⁺ cDNA. The cDNA was subcloned and sequenced with Sequenase (USB). Both strands were sequenced as well as all overlaps. Internal *Sall*, *XhoI*, *EcoRV*, and *XbaI* restriction sites are indicated. **b**, Northern blot analysis of *puc1*⁺ expression. Total RNA was prepared from *S. pombe*, run on a denaturing formaldehyde-MOPS gel, transferred to GeneScreen Plus and probed according to manufacturer's instructions (Dupont). A single-stranded probe corresponding to the 3' half of the cDNA was used. Markers were the 1.6-kb *cdc2*⁺ transcript²⁹ and the ribosomal RNAs. The sequence data reported will appear on the EMBL Nucleotide Sequence Database under the accession number X59154.



of *puc1*⁺ in *S. pombe*. Cells carrying a construct that overexpresses *puc1*⁺ on an episome were grown in the presence of thiamine and shifted to thiamine-free media. Wild-type cells overexpressing *puc1*⁺ are elongated, indicating that progression through the cell cycle is delayed (Fig. 4, upper panels). Flow cytometry showed that this delay occurred in G2.

This phenotype was highly accentuated when *puc1*⁺ was overexpressed in the mutant *cdc13-117* (ref. 8); *cdc13*⁺ is an essential gene encoding the mitotic B cyclin p56^{cdc13} of *S. pombe*, and the temperature-sensitive mutation *cdc13-117* leads to G2 or M arrest at the restrictive temperature^{8,26}. At the restrictive temperature *puc1*⁺ was unable to rescue the *cdc13-117* phenotype, indicating that *puc1*⁺ encodes a cyclin with a function quite distinct from the mitotic B-type cyclins. More interestingly, *puc1*⁺ overexpression was lethal to cells at the permissive temperature of *cdc13-117* (Fig. 4, lower panels). Cells became very elongated and arrested in the cell cycle with a single nucleus and a G2 DNA content. Thus overexpression of a G1-type cyclin in a strain harbouring a partially defective B-type cyclin leads to cell cycle arrest in G2. The simplest interpretation of this result is that the *puc1*⁺ cyclin directly interferes or competes

with the p56^{cdc13} B-type cyclin. Wild-type p56^{cdc13} is only modestly affected by overproduction of *puc1*⁺, leading to a slight delay in G2 and an increased cell size. But the mutant form p56^{cdc13-117} is unable to complete its role in the presence of an excess of *puc1*⁺. We suggest that *puc1* out-competes the mutant B-type cyclin in associating with other components of the cell cycle control network, effectively sequestering necessary proteins from their B-type cyclin-associated role.

These results establish that cyclins of the G1 class are not confined to *S. cerevisiae* but are found in other eukaryotic organisms, and confirm that both G1- and B-types of cyclins can be found in the same organism. Further investigation is required to establish fully the role of *puc1*⁺ in cell cycle regulation in *S. pombe*, as it is not clear whether or not it functions at G1 in the fission yeast. It is clear that *puc1*⁺ acts as a G1 cyclin in *S. cerevisiae*. Furthermore, that *puc1*⁺ seems to out-compete the p56^{cdc13} B-type cyclin suggests that these two classes of cyclin may differentially affect activity of the p34^{cdc2} kinase. It has been proposed that the p34^{cdc2} molecule acts as a cell cycle clock, indicating where the cell is in the cycle²⁷. It is possible that this timekeeping role is influenced by the formation

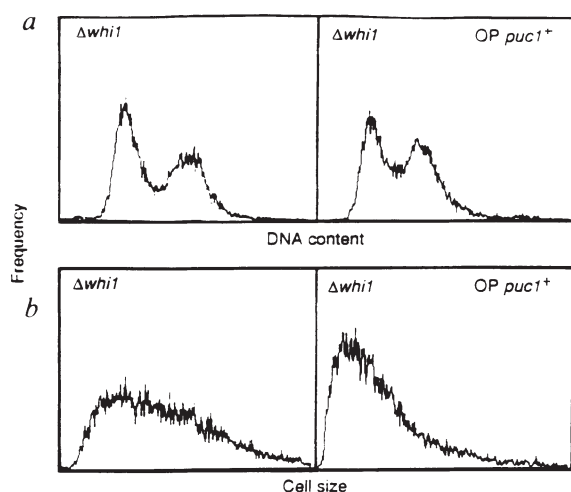
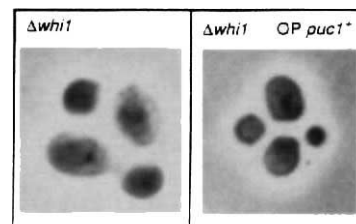


FIG. 3 Analysis of the effects of *puc1*⁺ overexpression in *S. cerevisiae* strain II32 ΔHpXB. Left, the strain transformed with a control plasmid (YE352). Right, the strain transformed with the 2 μm cloning vector, in which *puc1*⁺ is over expressed by the *ADH* promoter. *a*, Cellular DNA content,



as determined by fluorescence using flow cytometry. *b*, Cell size, as determined by forward angle light scatter using flow cytometry. Cell size measurements by forward scatter gave a mean of 103 for the control, compared with 65.7 for overproduction of *puc1*⁺ (relative units). We confirmed this with volume measurements in a Coulter counter, where the control strain had a mean cell volume (MCV) of 71.1 ± 1.2 compared with 38.5 ± 2.7 for the same strain overexpressing *puc1*⁺ (relative units). *c*, Phase contrast photomicrographs of the cells. Both strains were grown in selective media to an optical density at 595 nm of 0.4, fixed in ethanol and stained with propidium iodide for analysis carried out on a Becton Dickinson FACScan as described in ref. 30, or fixed in formaldehyde and photographed under phase contrast as described in ref. 28. Scale bar, 10 μm.

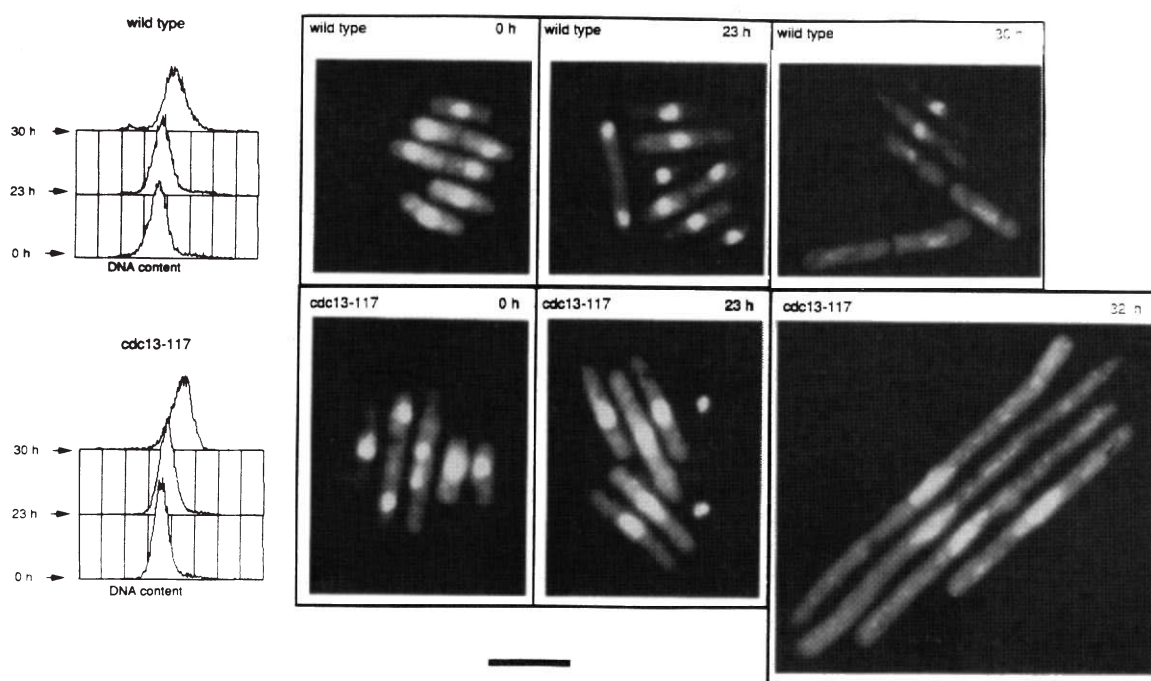


FIG. 4 Analysis of *puc1*⁺ overexpression in fission yeast. Top panels, flow cytometry of cellular DNA content and photomicrographs of the time course of induction of the overproducer plasmid pSLF59, expressing *puc1*⁺ under control of the *nmt1*⁺ promoter²⁴ in a wild-type strain (*h⁻ura4-D18 leu1-32*). The promoter takes ~15 h to begin derepression²⁴. As expression increases, the cells elongate and their growth rate is somewhat reduced. Bottom panels, flow cytometry of cellular DNA content and photomicrographs of the time course of induction of the same plasmid in a *cdc13-117* strain (*h⁻cdc13-117 leu1-32*). The cells become highly elongated (>60 μm at 40 h postinduction) and cannot form colonies. Cell length measurements of septated cells, taken with a drum micrometer, are as follows: wild type 0 h, $15 \mu\text{m} \pm 0.9$; wild type 23 h, $15 \mu\text{m} \pm 0.5$; wild type 30 h, $22 \mu\text{m} \pm 2.6$; *cdc13-117* 0 h, $18 \mu\text{m} \pm 1.0$; *cdc13-117* 23 h, $21 \mu\text{m} \pm 1.9$; *cdc13-117* 32 h, $39 \mu\text{m} \pm 8$. The promoter is switched off in 15 μM thiamine. The cells were shifted to selective medium without thiamine, to induce the promoter, and grown at 26–28 °C for the indicated times and kept in mid-logarithmic growth by constant dilution with the same medium. Cells were prepared and analysed by flow cytometry as described in the legend to Fig. 3; parallel aliquots were also fixed and stained with diaminophenylindole for fluorescence microscopy as described in ref. 28. Scale bar, 10 μm.

of different complexes with different classes of cyclin.

Note added in proof. Both types of cyclin have now been reported in *S. cerevisiae*^{31,32}.

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Solution structure of the major binding protein for the immunosuppressant FK506

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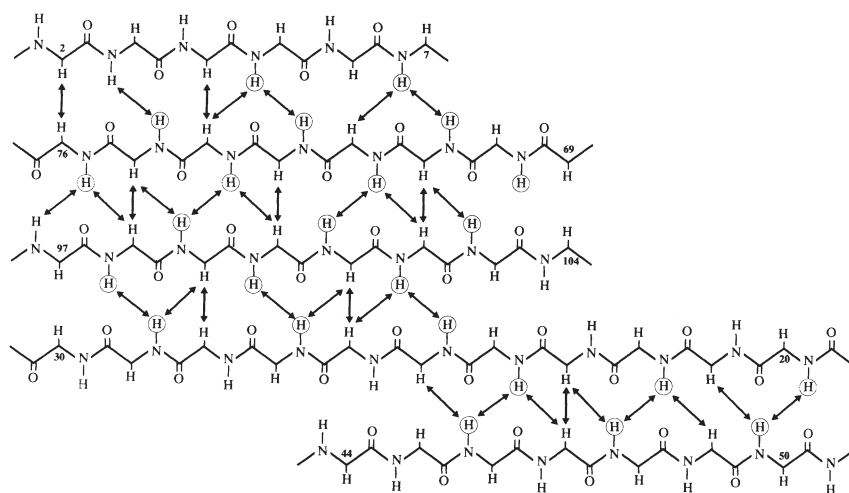
THE major FK506 binding protein (FKBP, relative molecular mass ~11,800; M_r 11.8K) and cyclophilin (M_r ~17K) belong to a class of proteins termed immunophilins¹. Although unrelated at the amino-acid sequence level, they both possess peptidyl-prolyl *cis-trans* isomerase activities which are inhibited by immunosuppressants that block signal transduction pathways leading to T-lymphocyte activation. FK506 and rapamycin strongly inhibit the peptidyl-prolyl *cis-trans* isomerase activity of FKBP, whereas cyclosporin A inhibits that of cyclophilin^{2–12}. The significance of this enzyme activity and the role of the immunophilins in immunoregulation is unknown^{1,13}. To understand better the function of the immunophilins and their interaction with inhibitors, we are investigating the solution structures of FKBP and FKBP-inhibitor complexes by multidimensional NMR methods. Here we report the solution conformation of FKBP, as generated by NMR, distance geometry and molecular dynamics methods. The regular secondary structure of FKBP is composed mainly of β sheet (~35%) with little helical structure (<10%). The hydrophobic

core of the molecule, containing the buried side chains of six of the protein's nine aromatic amino acids, is enclosed by a five-stranded antiparallel β sheet on one side, a loop and a short helix at residues 51–56 and 57–65, and an aperiodic loop at residues 81–95. Examination of the structure suggests a possible site of interaction with FK506.

Resonance assignments for the ¹H NMR spectrum of FKBP were done using a combination of the sequential assignment¹⁴ and main-chain directed approaches¹⁵. Stretches of the sequence in extended chain and helical conformations were identified from the characteristic patterns of through-space (<4.5 Å) ¹H–¹H connectivities observed in the two-dimensional nuclear Overhauser enhancement (NOESY)¹⁶ spectrum. β -Sheet supersecondary structure was assembled from both amide proton exchange data and NOESY data. Hydrogen bonding and NOESY patterns consistent with antiparallel alignment of five β -strand segments were identified and are shown in Fig. 1.

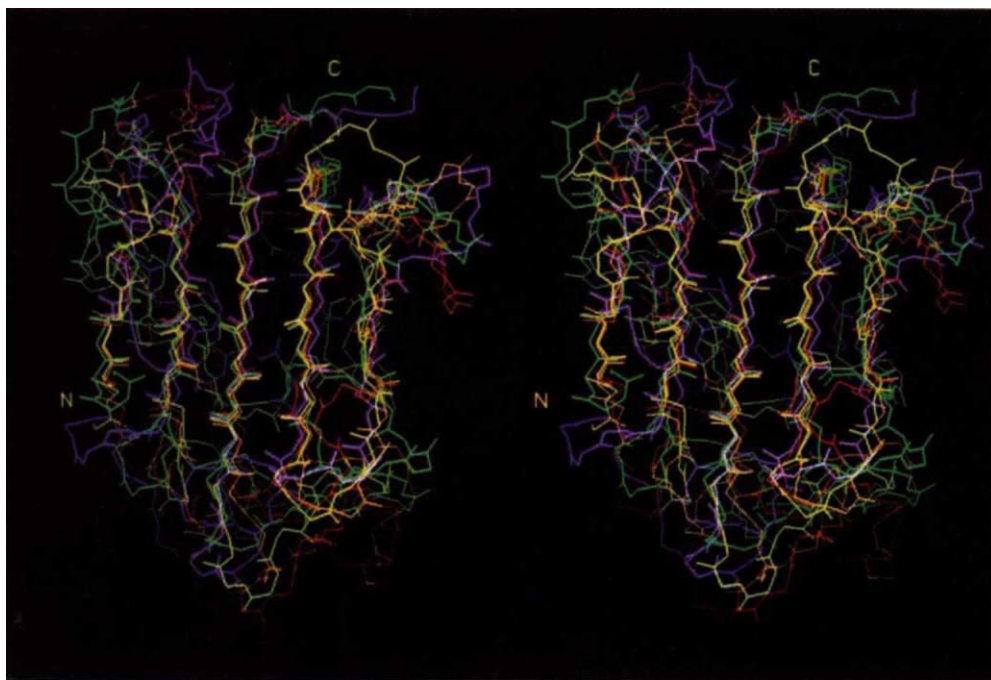
Solution structures were generated initially using the variable target function method in torsion angle space¹⁷. A set of 526 upper bounds on proton-proton distances were generated by calibration of the integrated intensities of NOESY cross-peaks versus the intensities of cross-peaks corresponding to known sequential distances. These constraints, together with 64 upper and lower bounds corresponding to hydrogen bonding constraints, were used as input for the distance geometry algorithm. Distance geometry structures having the lowest target function values (corresponding to fewest distance, dihedral angle, and van der Waals' constraint violations) were subjected to molecular dynamics refinement in the presence of the NMR constraints, using a heating and annealing protocol similar to

FIG. 1 Schematic diagram indicating NOESY connectivities used to define FKBP supersecondary structure. Arrows between adjacent strands indicate long-range NOEs observed for FKBP in 90% H₂O, 10% D₂O, 50 mM potassium phosphate buffer, pH 7.0 at 303 K with a NOESY mixing time (τ_m) of 60 ms. Circled amide protons indicate those observed to be in slow exchange, by their persistence in a two-dimensional J-correlated (COSY)²¹ spectrum acquired at 303 K immediately after exchange into a deuterated buffer medium of 100% D₂O, 50 mM potassium phosphate buffer, pH 7.0 (not corrected for isotope effects). Observed NOEs that could not be assigned unambiguously because of chemical shift degeneracy are not included. All ¹H NMR spectra required for resonance assignment were acquired using a Bruker AMX-500 spectrometer equipped with an X-32 computer. Data were processed using the Bruker UXNMR software. Two-dimensional data analysis, including peak assignment and integration, were carried out using the EASY (ETH Automated Spectroscopy)²² software. Data were collected at 303 K for protein samples at pH 7.0 and 5.5, and also at 290 K and 308 K for the protein sample with pH 7.0 to aid in assignment of overlapping peaks. All data presented were acquired from an 11.8 K FK506 binding protein purified from bovine thymus glands. The bovine and human proteins are highly homologous, containing only three conservative substitutions at Val 49 → Met, Asn 94 → His, and Ile 98 → Val (bovine → human). The protein was isolated by successive size-exclusion chromatography and hydrophobic interaction chromatography of calf thymus extracts. These methods require no thermal precipitation procedures to remove contaminating proteins, thus



avoiding the risk of inducing structural perturbation by thermal denaturation effects (J.A.T., M.J.F., J. R. Black and J. Boger, manuscript in preparation). The isolated protein was pure as judged by SDS-PAGE, by analytical isoelectric focusing, and by reversed-phase HPLC. N-terminal and internal sequence analysis showed clear identity with the reported sequence of bovine FKBP¹². The specific peptidyl-prolyl *cis-trans* isomerase and FK506 binding activities were equal to or greater than those previously reported^{8,9}, and all of the peptidyl-prolyl *cis-trans* isomerase activity was inhibitable by FK506 and was unaffected by cyclosporin A.

FIG. 2 Stereo view of five molecular dynamics-refined FKBP structures with lowest constraint energies (energy penalty due to residual NMR constraint violations) superimposed for best fit on backbone heavy atoms of β -sheet residues. The average r.m.s. difference from the average structure of backbone heavy atoms in the β sheet, for all backbone heavy atoms, and all atoms was 1.08 Å, 3.09 Å and 3.73 Å, respectively. Structures were determined using NOESY data (90% H₂O, 10% D₂O, 50 mM potassium phosphate buffer, pH 7.0) acquired at 303 K using a mixing time τ_m of 60 ms. Conversion of peak intensities into upper bounds was done as described by Guentert *et al.*¹⁷ using the program CALIBA. Constraints fell into the following categories: intraresidue (193), sequential (191), medium range (26) and long range (116). The resulting set of 526 constraints, with an additional 64 upper and lower bounds representing hydrogen bonding constraints was used as input for distance geometry calculations with the program DIANA¹⁷. Contributions to the target function from distance, dihedral angle, and van der Waals' violations were weighted equally. About 750 DIANA structures were generated, and the 20 structures with lowest target function values were subjected to molecular dynamics refinement using Discover (Biosym Technologies). Simulations were done using a consistent valence force field²³ to calculate the intrinsic strain energy, with an additional single-sided harmonic term added to the total energy for configurations which violate the NMR constraints. Explicit hydrogen bonding constraints used in distance geometry calculations were not used for molecular dynamics refinement. A distance-dependent dielectric constant ($1/r$) was implemented to reduce charges in the absence of solvent. Before dynamics, structures were energy minimized



for 1,000 iterations using a steepest descents algorithm in the absence of the NMR constraints. The system was then heated from 300 to 1,200 K in a linear fashion over the course of 1 ps using femtosecond time steps. During heating, the force constant K for the NMR penalty function was increased from 0 to 36 kcal mol⁻¹ Å⁻². The protein was allowed to equilibrate for 4 ps at 1,200 K, then cooled over a period of 6 ps to 300 K. Following dynamics, the molecule was subjected to an additional 2,000 iterations of conjugate gradients minimization in the presence of the NMR constraints. No structures shown had constraint violations greater than 0.5 Å.

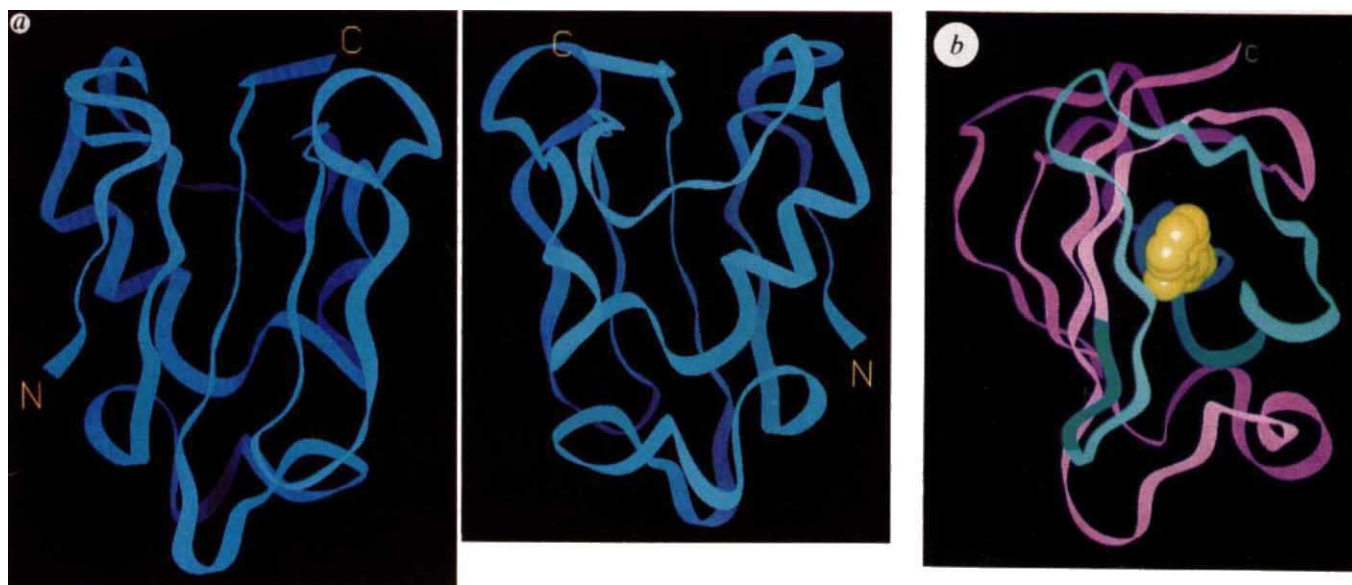


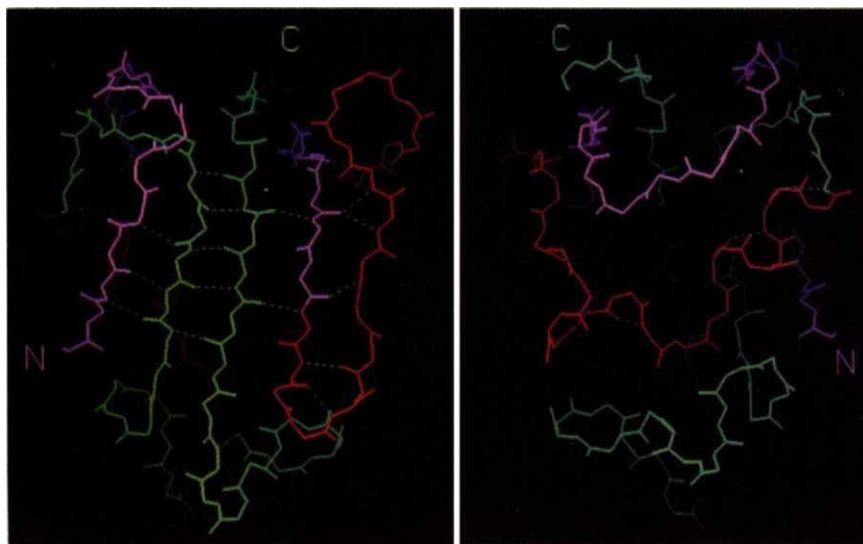
FIG. 3 *a*, Ribbon diagram showing the global fold of bovine FKBP. Alternative views, rotated through 180°, illustrating the β sheet (left) and helical regions (right). *b*, Ribbon diagram of a single FKBP solution conformation, illustrating a proposed FK506-binding cleft formed by the polypeptide chain at residues

32–55 (cyan). These and the flanking residues (29–31 and 56–65, blue) comprise the structural motif encoded by a single exon in the human genome (see Fig. 4). The heavy atoms of Trp 59 are shown in space-filling representation (yellow).

that described by Lee *et al.*¹⁸. Five FKBP structures with the lowest constraint energies (the sum of energy penalties due to residual constraint violations), and no violations greater than 0.47 Å, are shown in Fig. 2. Each of the five conformations shown satisfy all of the NMR constraints, have roughly equal

energies, and thus represent equally probable structures of FKBP. The superimposed structures are best defined in the highly constrained β -sheet region, particularly in the central region of the molecule. Overall, the β strands comprising residues 3–7, 20–29, 46–49, 71–77 and 97–103 exhibit an average

FIG. 4 Structural elements of single molecular dynamics-refined structure of FKBP are coloured according to genomic organization of the DNA sequences that encode them. Alternative views, rotated through 180°, illustrating the β sheet (left) and helical regions (right). Sequence data from human cDNA and genomic clones indicate that the sequence encoding FKBP amino acids 29–65 (red) is separated from that encoding amino acids 66–107 (green) by a 3.2-kilobase (kb) intron (D.A.P., J. A. Lippke, K. Hsiao & M. Benasutti, personal communication). Genomic information for the sequence encoding the first 28 amino-acid residues (magenta) of FKBP is incomplete at present. Two DNA oligomers 5'-AACAAAGCCCTTT-AAGTTTATG-3' and 5'-CATAAACTTAAAGGGCTT-GTT-3' were selected from published sequences of human FKBP cDNAs^{24,25} and synthesized on an Applied Biosystems 380A DNA synthesizer. These oligomers were radiolabelled with ³²P and used as probes under high stringency²⁶ to select two recombinant phage from a human T-cell cDNA library (Clontech). The cDNA inserts were purified, cloned into M13mp19 and sequenced by the dideoxy method²⁷. Both cDNA inserts contained an open reading frame that encoded FKBP. One cDNA insert was radiolabelled with ³²P and was used under high stringency as a probe to select recombinant phage from a human placental genomic library (Clontech). DNA was purified from four selected phage, digested with a variety of restriction endonu-



cleases, fractionated by agarose gel electrophoresis and transferred to a nylon membrane. The ³²P-labelled cDNA insert was used as a probe to select a 4.5-kb genomic fragment that was subcloned into pUC19 and sequenced by the dideoxy method.

r.m.s. difference from the average structure (backbone heavy atoms) of 1.08 Å. A helical region occurs at residues 57–66 and is reproducible in all structures. The loop in the region preceding this helix is not well localized and, at this resolution, has several conformations consistent with the NMR constraints. The same holds for other regions in the sequence which correspond to aperiodic secondary structure. These regions have the same overall fold among the family of structures, but are poorly defined locally due to a lack of NOESY data, and probably reflect a higher degree of mobility. Overall, the secondary structure content observed by NMR at this resolution agrees well with secondary structure estimates from circular dichroism measurements (J.A.T., unpublished observation). Figure 3a illustrates the global fold of FKBP from several perspectives.

The hydrophobic core of the molecule is defined by numerous NOE contacts, and contains most of the aromatic sidechains, including Tyr 26, Tyr 82, Phe 36, Phe 46, Phe 48, and the single Trp residue at position 59. The position of Trp 59 in the hydrophobic core is consistent with intrinsic fluorescence measurements, which suggests that this residue is in an extremely apolar environment (J.A.T. and M.J.F., manuscript in preparation). Furthermore, changes in the fluorescence parameters, on binding of FK506, suggest either the direct interaction of FK506 with the Trp sidechain or perturbation of the Trp environment due to longer-range conformational effects of drug binding (J.A.T., M.J.F. and P.R. Connelly, unpublished observations). Supporting this is the fact that the pipicolinyl group of bound FK506 is close to several benzenoid protons of Trp 59 in human recombinant FKBP¹⁹. Examination of the solution structures presented here reveals a possible FK506 docking site in the cleft formed by the polypeptide chain at residues 30–55 (Fig. 3b). Binding of FK506 near these residues is consistent with site-directed mutagenesis studies of Trp 59 in human recombinant FKBP, which show that alteration of this residue to Leu substantially decreases the affinity of the mutant protein for FK506 relative to wild-type protein (D. J. Livingston, personal communication).

The solution structure of FKBP also presents striking organizational elements when considered in conjunction with DNA sequence information (Fig. 4). Amino acids 29–65 comprise the loop and helical region that enclose one face of the FKBP hydrophobic core and residues 66–107 constitute the two central strands of the β sheet. Comparing human complementary DNA

to human genomic sequence does not yet identify the genomic element encoding the 28 N-terminal residues of FKBP but does reveal that residues 29–65 and 66–107 are encoded by separate exons. Since genes of complex eukaryotes can be viewed as collections of exon modules that code for structural elements, functional domains or folding regions of proteins²⁰, it is tempting to speculate that one or both of these FKBP exons may be used to encode structural motifs within other immunophilins. The structures presented provide a structural basis for the understanding of drug-immunophilin interactions. The X-ray crystal structure of bovine FKBP has been solved by molecular replacement using the NMR structures presented here and will be presented elsewhere (M. M. Yamashita, J.A.T., J.M.M., M.J.F. and M. A. Navia, manuscript in preparation). □

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Displacement chromatography

S. M. Cramer

Displacement chromatography is a multicomponent preparative chromatographic technique that enables simultaneous concentration and purification of biomolecules.

THERE has been a recent resurgence of interest in displacement chromatography (DC) as a preparative separation technique for biomolecules¹⁻³. This nonlinear mode of chromatographic operation offers tremendous potential for simultaneous concentration and purification of biomolecules. In contrast to overloaded elution chromatography, which can often result in significant tailing of the peaks and dilution of the feed⁴, DC can produce sharp boundaries and concentrated products during the separation process. Because the process takes advantage of the nonlinearity of the adsorption isotherms, a chromatographic column operated in the displacement mode can process higher feed loads, enabling researchers to purify large amounts of material with relatively small chromatographic columns¹⁻³.

Displacement chromatography can be carried out using existing chromatographic systems with minor modifications to enable the sequential perfusion of the column with carrier, feed, displacer, and regenerant solutions. In DC, a front of displacer solution travelling behind the feed, drives the separation of the feed components into adjacent pure zones. The displacer is selected such that it has a higher affinity for the stationary phase than any of the feed components. The order of the displacement zones corresponds to the increasing affinity of the components for the stationary phase. The concentration of each component in the final displacement train is determined solely by its adsorption isotherm and the concentration and isotherm of the displacer as shown in figure 1. Thus, when the concentration of the feed components are less than those dictated by the final isotachic conditions, DC results in significant concentration of the feed components during the separation. Upon the emergence of displacer, the column is regenerated with an appropriate solvent followed by re-equilibration with the carrier.

Practical applications

Although the physico-chemical basis of DC was established by Tiselius in 1943 (ref. 5), it is only recently that DC has been actively investigated for the simultaneous concentration and purification of biomolecules¹⁻³. Displacement chromatography has been used successfully for the separation of a wide variety of biomolecules on diverse stationary-phase materials. Amino acids have been purified by DC on ion exchange adsorbents using sodium hydroxide⁶ and benzyltributylammonium chloride⁷ (BTBA) as displacers. Racemic mixtures of dansyl-leucine have

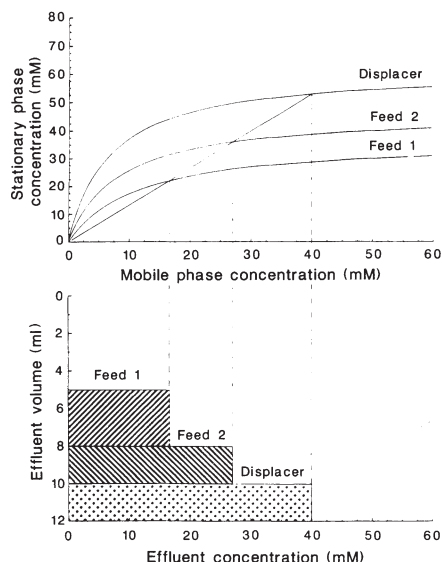


FIG. 1 Schematic representation of isotherms of the feed components and the displacer along with the operating line and the corresponding fully developed displacement train (from ref. 19).

also been separated by DC on a β -cyclodextrin chiral support⁸. A scale-up procedure has recently been reported for the purification of 38 grams of amino acid derivatives by DC on normal-phase systems⁹.

A variety of peptides have been separated on reversed-phase columns using DC¹⁻³. Enzymatic peptide synthesis mixtures have been purified using 2-(2-butoxyethoxy) ethanol (see Fig. 2), decyltrimethylammonium bromide, and cetramide as displacers¹⁰. Peptides have been purified by DC at elevated flow rates, resulting in the simultaneous purification and concentration of 40 milli-

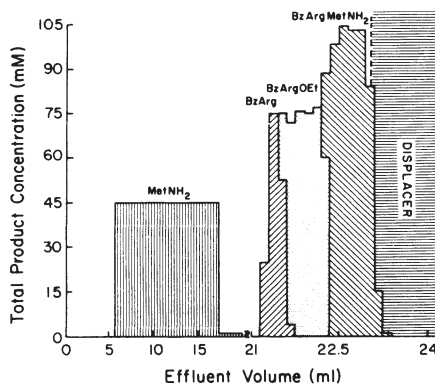


FIG. 2 Displacement chromatogram obtained in the purification of Bz-Arg-Met-NH₂ from 15 ml of feed using 247 mM 2-(2-butoxyethoxy) ethanol as the displacer on a 250 $\times$ 4.6-mm I.D. column packed with 10 μ m C-18 (from ref. 10).

grams of peptides in 8.3 minutes on an analytical reversed-phase column¹¹. Biologically active α - and β -melanocyte stimulating hormones have been isolated from complex mixtures by DC using benzyldimethyldodecylammonium bromide as the displacer on analytical reversed-phase columns¹². Displacement chromatography has also been used for the purification of peptide diastereomers¹³. Synthetic peptides used in an ELISA immunoassay of anti *Plasmodium vivax* sporozoite antibodies have recently been separated by DC on a reversed-phase system¹⁴.

An automated recycle enzyme reactor-displacement chromatograph system has been developed for the synthesis and purification of dinucleotides¹⁵ and dipeptides¹⁶. These tandem reactor-separator systems may have significant potential for the preparation of complex biochemical substances in the laboratory. Nucleotides and nucleosides have been purified using butanol and BTBA, respectively, as the displacers on a reversed-phase system⁷. Several antibiotics have also been purified using displacement chromatography on reversed-phase systems¹⁻³.

One of the most pressing challenges to the biotechnology industry is the efficient purification of proteins. Several research groups are currently involved in the development of novel protein displacement chromatographic systems. Anion exchange adsorbents have been used successfully in the displacement mode for the separation of proteins using carboxymethyl dextran¹⁷ and chondroitin sulphate¹⁸ as displacers. Proteins have also been purified using cationic polymers¹⁹ and DEAE-dextran of various molecular weights²⁰ as displacers in cation exchange systems. In addition, two kilodalton polyvinyl sulphonic acid has recently been identified as a suitable displacer for proteins on anion exchange systems. Displacement chromatography has been used in concert with frontal chromatography and an intermittent column flush to separate relatively large quantities of proteins²². These experiments have demonstrated that DC may be useful for the simultaneous purification and concentration of proteins.

The effects of crossing adsorption isotherms on displacement behaviour has been examined with protein displacement systems³. While these experiments indicated that crossing isotherms resulted in mixing of the components, appropriate changes in the adsorptive conditions readily 'un-crossed' the isotherms, resulting in successful dis-

placement separations. A recent theoretical study²³, which generates multicomponent isotherms within the framework of the ideal adsorbed solution theory, promises to shed light on this phenomenon.

Displacement chromatography has been successfully scaled up with respect to particle and column diameter²⁴. The ability to carry out DC with relatively large particle-diameter systems is expected to have a significant impact on the economics of the process.

Cyclodextrin-silica chiral columns have been used in the displacement mode for the separation of a variety of compounds⁸. Immobilized metal affinity chromatographic systems have recently been used in the displacement mode for the simultaneous concentration and purification of proteins²⁵. The use of these unique adsorbents in the displacement mode has the significant advantage of combining the selectivities of these systems with the high throughput and purity obtained in DC.

Displacement chromatography has recently been used in concert with continuous-flow fast atom bombardment-mass spectrometry²⁶ to overcome sample capacity limitations usually imposed by directly coupled liquid chromatography-mass spectrometry systems. Continuous annular chromatographic systems have also been operated in the displacement mode²⁷. This process combines the high throughput of DC with the ability to operate annular chromatographic systems continuously.

There is presently much activity in the area of modelling non-ideal displacement chromatographic systems²⁸⁻³³. However, an accurate determination of the parameters involved in these models and an understanding of the concentration dependence of these parameters, is not well in hand.

What the future holds

While significant progress has been made in the theory and practice of DC over the past 10 years, much work remains to be done before the process is widely adopted for the downstream processing of biopharmaceuticals. Areas of displacement chromatography requiring active research include the development of novel non-toxic displacers, detailed studies of column regeneration, 'case studies' that test the resolving power of DC under 'real-life' conditions, extension of DC to alternative adsorptive systems, and theoretical work on the modelling and optimization of displacement chromatographic systems. Although DC is still in the development phase, researchers who are actively involved in preparative bioseparations may find it a powerful technique, particularly when it is used at the early stages of the downstream bioprocessing scheme. □

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Tools for separation

This week's look at what's new for the chromatographer features software that is designed to take the guesswork out of HPLC methods development and a high-capacity autosampler with refrigeration option for thermally-sensitive samples.

With the introduction by Fisons of the Carlo Erba SFC 3000 **supercritical fluid chromatograph**, researchers have the choice of supercritical fluid chromatography (SFC) and gas chromatography (GC) in a single instrument (*Reader Service No. 101*). The SFC 3000 offers SFC with packed, capillary and micropacked columns, and capillary GC. At the heart of the system is the SFC 300 pump, a 150-ml capacity, pulse-free syringe pump for delivery of mobile phase. Although the pump, which can be set to operate in constant flow or constant pressure modes, has been developed specifically for SFC, it can handle a variety of liquids, making it suitable for other applications, says Fisons. Gradient elution is possible using up to four pumps, and flow rates can be controlled from 1 $\mu\text{l min}^{-1}$ to 7 ml min^{-1} . Because of the different demands of SFC and GC, two independent injection systems are used: a fast, thermostatted and easily automated system for SFC and an on-column injector with secondary cooling for capillary GC. Oven temperatures can be set from near ambient to 450 °C in 0.1 °C min^{-1} increments. The SFC 3000 system can either be programmed through the built-in keypad or via a remote PC.

The new multimode, high-capacity **fraction collector** from Gilson Medical Electronics, the FC 204, can handle up to 432 10-mm tubes (*Reader Service No. 102*). For added flexibility, various racks are available.



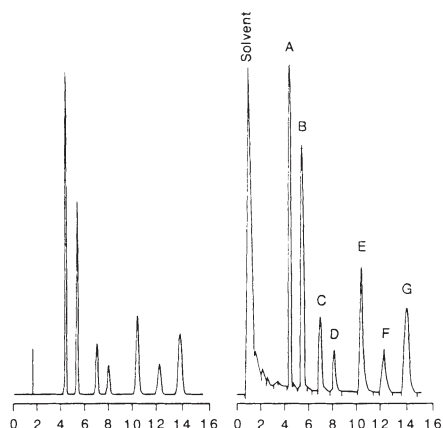
FC 204: for time, drop or peak collection

For preparative applications, the FC 204 can hold 176 18-mm tubes. For semi-preparative and analytical HPLC applications, the collector can accommodate 240 13-mm tubes and 432 10-mm tubes, respectively. The FC 204 can be programmed for time, drop or peak collection. In addition, up to 10 collection windows can be defined in any mode, says Gilson. During operation, users can manually control tube advance and drain functions. When used with Gilson's HPLC systems, the fraction collector can be controlled by a system controller with Gilson software. It can also be used with other systems. For example, in low pressure LC systems, it can act as a system controller, providing start/stop control of pumps and chart recorders. Metal drive components make the FC 204 suitable for cold-room use.

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Software options

Take the trial and error out of HPLC methods development, says PhaseSep, with the company's **HPLC optimization software packages**, which are designed for IBM and compatible PCs (*Reader Service No. 103*). Three stand-alone packages are available. The basic Hipac B package optimizes normal phase, reverse phase, pH, ion-pair and ion-exchange isocratic separations. Optimal values for parameters such as column length,



Simulated chromatogram (left) using Hipac optimization software versus actual chromatogram (right).

particle size and flow rate can be determined. In addition, the software identifies the optimum phase composition for maximum resolution in minimum analysis time, says PhaseSep, producing a simulated chromatogram at this composition. Hipac G can be used to optimize the elution gradient for single or multi-step binary reversed-phase separations. The program will provide a simulated chromatogram of the optimum conditions overlain with the optimum gradient. A tabular print-out of, for example, retention times and resolution factors, is also provided. Hipac TQ can be used to optimize solvent conditions for ternary and quaternary separations, as well as pH and organic modifier. Free demonstration disks are available on request.

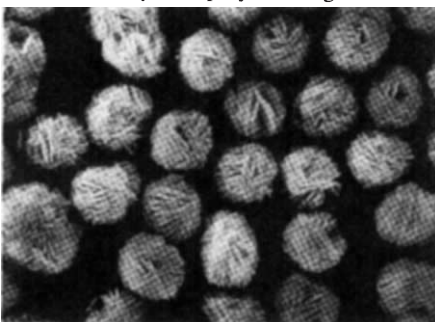
Pyramid Chromatography Manager from Axxiom Chromatography is a user-configurable **data acquisition and analysis system** that operates in the Microsoft Windows 3.0 multitasking environment (*Reader Service No. 104*). Chromatographers can customize all Pyramid displays, functions and modes to match the requirements of any specific application in GC, HPLC, IC, SFC and CZE, says Axxiom. Pyramid's optional interfaces allow the user to control a system's components, that is, pumps, detectors, autosamplers, fraction collectors, GC ovens and accessories. Two versions of Pyramid are available for managing one or two chromatographs: Pyramid I accepts two simultaneous detector inputs and controls one complete chromatography system via a single interface module, and Pyramid II accepts up to four simultaneous detector

inputs from one or two independent systems via two interfaces.

Column chemistries

Supelco's Suplex pKb-100 **HPLC column** is designed to virtually eliminate interactions between basic sample components and residual silanol groups on the packing surface (*Reader Service No. 105*). Even basic compounds can be analysed under mobile phase conditions of neutral pH and low ionic strength, without amine modifiers, says Supelco. The Suplex column is designed to provide improved resolution, peak shape and detection when compared to conventionally deactivated or non-deactivated columns. In addition, all the benefits of reversed-phase HPLC on porous silica are retained, says the company.

Unisphere-C18 **media** combines the selectivity needed to purify peptides and proteins with the pH stability that provides flexibility and long column life, says Biotage (*Reader Service No. 106*). This alumina-based support consists of fused porous plates coated with a thin layer of polymer to give them a



Unisphere-C18 media — see Biotage

typical C18-like surface chemistry. The coating provides the surface chemistry selectivity with no nonspecific adsorption sites, says Biotage. The pH stability range of Unisphere-C18 media is 2–13. The macroporous platelet structure combined with a traditional microporous surface is designed to provide mechanical strength and high loading capacities. Several column sizes are available.

The Nest Group has introduced a new family of **non-porous HPLC chemistries**, which are intended to provide ultrafast protein analyses with quantitative recovery even at nanogram levels (*Reader Service No. 107*). The non-porous resin in the TSK-Gel NPR columns limits diffusion to surface binding, making protein adsorption/desorption fast. Ten-minute gradients are possible, as compared to 30–60 minutes with porous supports, says Nest. TSK-Gel NPR columns are available in ion exchange, reversed-phase and hydrophobic interaction chemistries. The columns have chemical and mechanical stability and can be used from pH 2–12. Fouled columns can be cleaned with NaOH. Suggested applications include monitoring the kinetics of protein

production and mutations, fermentation mixtures and protein refolding studies.

Automated sample prep

Applied Chromatography Systems offers a new **autosampler and derivatization system**, called the 750/160, which has a 160-sample capacity and requires as little as 5 µl of sample (*Reader Service No. 108*). The



ACS's autosampler with 160-tube capacity.

750/160 system can be set to inject sample volumes from 1–250 µl. The system features a handy vial shaker to assist with dilutions, standard additions and pre-column derivatization for amino acid determinations. Injection volumes and the number of injections per vial can be selected for each vial and are programmed via the touch-sensitive keypad.

A wide range of injection volumes (0.5–400 µl) and a variable sample capacity (50-, 70- or 100-vial racks) are features of the AS-2000 compact **autosampler** from Hitachi Instruments (*Reader Service No. 109*). As little as 0.5 µl of sample can be injected from a 6 µl total sample volume. Other features include three injection methods (full loop, cut volume and all volume), five syringe speeds and a needle wash cycle that is designed to eliminate carryover. The selectable parameters for routine analysis include: number of vials to be analysed, number of injections per vial, injection volumes, analysis times, number of standards, number of unknown samples between standards, and linking of different sets of parameters.

Varian offers an air-cooled refrigeration option to the LC Star 9100 **microprocessor-**

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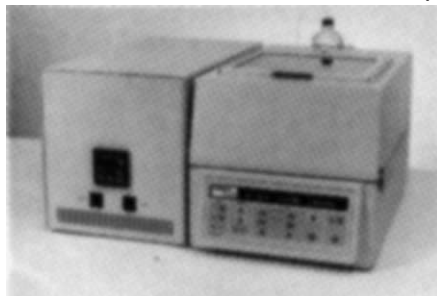
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controlled autosampler, which is designed for laboratories running high volumes of thermally-sensitive compounds (*Reader Service No. 110*). Even with the refrigeration option, the 105-sample capacity is retained, says Varian. The 9100 autosampler can be integrated with the Varian LC Star system with its Star Workstation and Microsoft Windows operating environment, or it can be interfaced with other laboratory



Refrigerated option to Varian's autosampler.

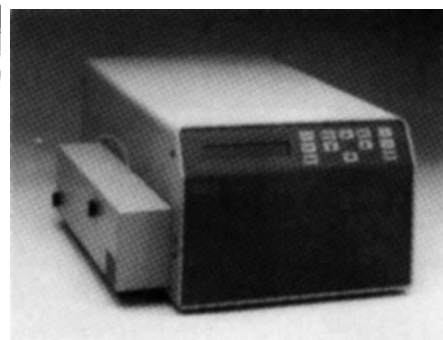
equipment. Standard Valco or Rheodyne injectors provide injection volumes in full- or partial-loop modes. With Automix vial-to-vial liquid transfer and mixing, extractions, dilutions, derivatizations, addition of internal standard, and initiation and quenching of reaction kinetics can be automated. The refrigeration option can be retrofitted to existing equipment. Prices for the LC Star 9100 autosampler range from \$11,550 to \$15,900 (US).

Detection limits

Ciba Corning Analytical is distributing Jasco's Model 821-FP microprocessor-con-

trolled **fluorescence detector**, which offers time programming of both the excitation and emission wavelengths and a slit width that is variable in two steps — 18 and 30 nm (*Reader Service No. 111*). The detector uses a 150-W high-energy xenon lamp housed in a sealed lamp house to enhance sensitivity. As defined by the signal-to-noise ratio of the Raman band of water, the detector has a sensitivity that is better than 120 using an excitation wavelength of 350 nm, says Ciba Corning. In order to maximize the energy throughput and prevent chromatic and focusing aberrations, only reflective optics are used, says the company. And, to eliminate 'ghost' artefacts and reduce stray light, holographic concave gratings are used in both the excitation and emission monochromators.

Bioanalytical Systems has introduced a new **fluorescence detector**, the FL-45, which features dual stepper motors for monochromator control of both excitation and emission wavelengths (*Reader Service No. 112*). Up to 10 different methods can be stored in the instrument's non-volatile memory. Both the excitation and emission wavelengths are programmable in 2-nm steps from 200–650 nm, with an option for an emission wavelength range of 200–800 nm. The \$8,495 (US) FL-45 uses a pulsed xenon lamp with a selectable flash rate: 100 Hz for enhanced sensitivity, reduced to 20 Hz when less sensitivity is required to prolong lamp, life, says BAS. The 2 × 4-mm flow cell has a 3.5 µl illuminated volume. BAS says the time-programmable FL-45 fluorescence



Fluorescence detection from 200 to 650 nm.

detector has a sensitivity of less than 100 femtogram of anthracene.

Systems for sale

LC Module 1 is the newly released **complete HPLC system** from Waters Chromatography Division (*Reader Service No. 113*). The pump module allows the blending of up to four solvents for the formation of isocratic mobile phases or gradients. UV/visible detection over the wavelength range of 190 to 600 nm is provided. Wavelengths can be switched during the run. In order to speed up routine analyses, the system features an autosampler with a 96-sample capacity. Up to 15 methods can be stored. Compatibility with Water's range of data workstations and integrators, provides a means of fully documenting the operating conditions, methods and results.

The DX-300 Series from Dionex can be configured for **gradient ion chromatography or HPLC** (*Reader Service No. 114*). The inert, biocompatible polyetherether ketone (PEEK) construction of all flow paths allows the user to select any buffer, pH, or reversed-phase solvent. Depending on the analytes to be determined, users can choose from conductivity, amperometric, UV and fluorescence detectors, and from a range of Dionex ion exchange and reversed-phase columns. Microbore columns can be used at flow rates as low as 0.01 ml min⁻¹. For semi-preparative applications, 9-mm ID columns can be used at flow rates up to 10 ml min⁻¹. The real-time electronic pulse damping provides a smooth, pulse-free flow that allows even flow-sensitive detectors, such as amperometric and mass spectrometers, to operate with maximum signal-to-noise ratios, says Dionex. Complex samples can be separated with the help of eight preprogrammed exponential gradient curves. In addition, users can store up to 10 gradient programmes for frequently-used methods. The AI-450 Chromatography Workstation, which provides complete automation, is optional. □

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